Scale up of the microbial N-dealkylation of drug intermediates using Cunninghamella bainieri

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Summary

The culture and scale up of the fungus Cunninghamella bainieri was investigated. Reproducible growth of the fungus was produced when using a spore inoculum in the presence of 0.02% w/v tween 80 in shake flasks. Spore concentration, spore age and antifoam B did not have any easily detectable effect on growth rate or growth form.

Codeine was converted to norcodeine in shake flasks when presented as a 1 mM solution of codeine phosphate. The efficiency of the conversion was approximately 12%.

Scale up was carried out in a 5.5 l working volume airlift fermenter and a 9.5 litre working volume stirred tank fermenter. Adjustment of the internal arrangement of the airlift fermenter (lowering the gap between the draught tube and the fermenter bottom and removing the draught tube support ring, and the replacement of the sintered sparger with four orifices, resulted in the production of pelleted fungal growth.

Very little suspended growth was produced in the stirred tank fermenter, the growth was attached to many points within the fermenter. Removal of one attachment point resulted in heavier growth at another point.

In both reactors fungal pellets or attached growth produced a smaller surface area to volume than found in the shake flasks, and norcodeine production was much lower than in shake flasks (almost unmeasurable compared to 12% conversion in shake

flasks).

Glucose uptake in the airlift reactor was always more rapid than in the stirred tank reactors and it was concluded that the airlift fermenter was superior to the stirred vessel for the submerged culture of C. bainieri.

For my parents, who made University possible and Brenda who has put up with my insomniac ways.

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CONTENTS

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Microorganisms are capable of carrying out a very large number of chemical reactions on a wide range of organic and inorganic chemicals.

Many of these reactions are essential to the survival of the cells and include the TCA cycle, the production of sugars from carbon dioxide and light energy and the breakdown and synthesis of complex organic or organometallic compounds. These reactions are extensively covered in standard textbooks such as Lehninger (67).

Other reactions may only occur when induced, such as enzymes used to respond to a new nutrient source such as lactose (67).

Further reactions may be carried out of the end of the life of the cell when a state of unbalanced growth is occurring or when various nutrients are limited. These are the growth conditions in which many antibiotics are produced (2,29,80).

This ability of microbes to produce or degrade chemicals has been exploited for many years. Until recently the uses have been mainly food orientated,using microbes to produce beer, wine, vinegar, cheese, yoghurt and bread (4). Later developments include the use of nitrogen fixing bacteria in legumes as part of crop rotational procedure and deliberate sewage treatment

strategies.

Most of these processes can be carried out without any real understanding of the microbiology of the process. It is a long step from realising that clover helps to keep land fertile to tracking down the rhizobium bacteria within the root nodules of the clover.

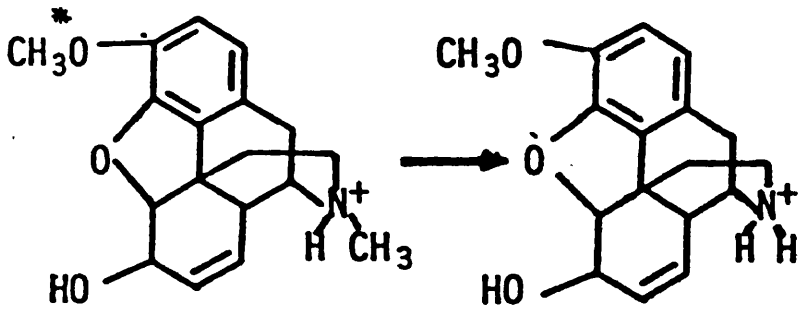
Over the last century more uses have been made of microbes. Enzymes for the food industry, antibiotics, microbial protein and genetically engineered products have been produced (4,29,112). The replacement of chemical reactions in some steps of steroid production has also been carried out using microbes (2,58).

The aim of this study was to investigate the possibility of using a filamentous fungus to N-dealkylate drug intermediates. A biological system was used to carry out the N-dealkylation of drug intermediates because, as is often the case, the products of biological systems are difficult to synthesise and may utilise dangerous chemicals for their production. Polypeptides such as insulin and interferon are both such compounds and efforts to produce them easily and in large quantities are concentrated on genetic engineering and cell culture techniques (2). Another approach is the use of microbes containing one specific enzyme to carry out a single step in a chain of reactions. Steroid conversions are a good example of this approach (2,58,108,112).

It is envisaged that N-dealkylation of drug intermediates may be used as a single step in such a

Figure 1.1

A Diagram of the Codeine and Norcodeine Molecules Showing the Sites of 'N' and 'O' Dealkylation



* = site of O-Dealkylation

+ = site of N-Dealkylation

chain of reactions where the substitution of an 'N' function is required. Although this reaction can be carried out by chemical methods the conditions used include low pH, boiling reagents and toxic reagents (108,109). A three fold scale up (8g to 21g) of the N-dealkylation of codeine to norcodeine using these methods resulted in a drop of yield from approximately 60% (44) to 16% (see section 2.2.4).

Biological conversion offers a chance to achieve conversion on a larger scale without the risk inherent in using the conditions mentioned above. Sewell showed conversion of a range of compounds by Cunninghamella spp (108). A model compound containing both 'O' and 'N' alkyl groups was chosen for use in further investigations. This was the opiate codeine (fig 1.1), which enabled the specificity of the N-dealkylation reaction to be studied. The use of N-dealkylated compounds by the pharmaceutical industry indicates that an efficient microbial N-dealkylation system may have commercial potential (109).

Work by Gibson (44) showed that the N-dealkylation reactions under study are probably catalysed by cytochrome P-450 monooxygenases (cyt P-450's). These enzymes show an absorption peak around 450nm, when the difference spectra of reduced enzyme reacted with carbon monoxide and unreduced enzyme are studied (115). Gibsons findings complement the work of Ferris et al. (39,40) who identified cyt P-450 as being

responsible for catalysing several reactions in Cunninghamella bainieri(C. bainieri).

The first step in identifying a potentially useful organism is screening to test whether the desired reaction will be carried out. This is normally done using as small a scale as possible to maximise the number of cultures tested. Once the best small scale cultures have been identified they are tested to see if they can be scaled up to a useful size and then optimised at the large scale. The problems presented by scale up can be considerable.

A large number of bacteria and fungi were screened by Sewell (108) and the filamentous fungi of the Cunninghamella Spp were found to be most efficient at carrying out the N-dealkylation reaction. Further experiments were carried out by Sewell to try and determine the best medium for growth and conversion. Most of Sewells work was carried out in shake flasks and a few runs in the 12 l L.H. stirred tank fermenter (STF) were also reported (108). The aim of this project was to obtain preliminary data for scale up using shake flasks, a 5.5 l volume airlift fermenter (ALF) and the STF. Problems such as standardising inoculum procedures, attempting to control growth form and reducing variability of results were investigated. It was then hoped to be possible to optimise the conditions for N-dealkylation using batch and continuous fermentations in various culture vessels.

1.2 Cytochrome P-450 Systems

Cytochrome P-450 systems are very widespread and the rat liver cyt P-450 system has been very widely studied because of its activity on xenobiotic compounds(114,115).

Prokaryotes are not frequently found to contain cyt P-450's and those prokaryotes which have been shown to possess cyt P-450's display activities with a limited range of substances e.g. Bacillus megaterium (7) and Pseudomonas (114,115)

Many eukaryotes have been shown to perform cyt P-450 mediated reactions and a wide range of substances may be used by a single organism (31,39,40,114,119).

Several eukaryotic systems, especially mammalian livers and several Cunninghamella spp have been shown to carry out cyt P-450 mediated reactions on an especially large number of substances. Evidence suggests the involvement of several cyt P-450's in these systems - not one non specific enzyme. A recent review of literature (114) suggested the following:

1. The specificity of cytochrome P-450's may alter depending on the inducer used.
2. Different inducers may induce enzymes with different spectra (i.e. a peak at 448nm and not 450nm)
3. SDS electrophoresis of rabbit liver microsomes revealed 8 distinct bands after induction with different compounds.
4. The substrate specificity of cyt P-450's from

different tissues in the same mammal vary.

It is also known that two yeast strains which carry out N-dealkylation were not induced to produce cyt P-450 by liver cyt P-450 inducers (see section 1.3). There was also no evidence of N-dealkylation by the yeasts.

1.3 Cytochrome P-450's from yeast and filamentous fungi

Sewell (108) and Gibson (44) provided strong evidence that the monooxygenase system found in C. bainieri C43 was a cyt P-450 system. A literature survey was carried out to try and correlate information about cyt P-450's from filamentous fungi and yeasts (see below). Not all the systems quoted have been shown to possess cyt P-450's but they share many properties with the other systems mentioned.

The properties of the systems studied were:-

i All the systems studied were capable of being induced or derepressed to some extent (12,30,31,46,47,65,68,76,79,101,104,114,118,126,136).

ii Filamentous fungi and yeasts used cyt P-450's to metabolise a large number of substrates including n-alkanes (104),aromatic hydrocarbons (21) and various drug substances such as phenobarbitols and 3 methylcholanthrene (3-MC) (114,115).

iii Dissolved oxygen tensions of 10-15% of atmospheric levels were shown to be optimum for the induction of cyt P-450's (27,82).

iv Optimum dissolved oxygen tensions for the expression of cyt P-450's appear to be about 30% of atmospheric oxygen tension (27,82). As some of this work used fungal pellets the actual optimum dissolved oxygen tension may be much lower than the figure quoted.

v Glucose concentrations of the level normally used in microbiological media prevent the formation of cyt P-450's (eg 30,31,114,115). By growing Saccharomyces cerivisiae in conditions which prevent the formation of mitochondria i.e. aerobically in high (20%) glucose media, or anaerobically, Wiseman(135,136) has shown the importance of cyclic adenosine monophosphate (cyclic AMP) in cyt P-450 regulation in at least one case.

vi One case is known of the conversion of cyt P-450 to cyt P420 (a form inactive in the bioconversions in which we are interested) which was caused by the incubation of Candida tropicalis strain 101 in the presence of antifoam B (31). This antifoam is a silicon polymer. No other reports of detrimental effects of antifoams on cyt P-450's have been found.

It is necessary to qualify the second point by stressing that the range of substrates attacked by cyt P-450's from different strains of organism may vary greatly. At one end of the scale Cunninghamella strains have been found to be capable of metabolising a large variety of compounds by using cyt P-450's and seem to carry out almost as many reactions as the mammalian liver

(115). On the other end of the scale some organisms posess cyt P-450 systems which are only capable of acting on a much smaller range of compounds. Three examples are mentioned here:

a) Candida tropicalis strain 101 has been reported to contain a cyt P-450 system which is induced by straight aliphatic chains having two methyl groups and one methyl and a phenyl or alchohol group at each end and being 10 or more carbon atoms long. Several compounds were unsuccessful as inducers, these included two classic hepatic cyt P-450 inducers (phenobarbitol and 3MC) and pristane, a branched hydrocarbon (46). Other strains have been shown to have a much wider specificity (31).

b) Saccaromycopsis lypolytica, a yeast which grows on n-alkanes will hydroxylate n-hexadecane, n-alkanes and lauric acid very well. No O or N-deaklylation of drug compounds has been observed in this organism (76).

c) The narrow specificity of cyt P-450 mediated reactions in this group of eukaryotes may not be limited to yeasts. Circumstantial evidence that a specific system possessed by the filamentous fungi Fusarium oxysporum f.pisi, F.solani f.pisi and Nectrina haematococca (Fusarium solani) was a cyt P-450 system is quite strong. This system was involved in the breakdown of pisatin (a flavenoid type of phytoalexin) (30,130).

Once a suitable strain of organism has been

selected the effects of the carbon source and oxygen are obviously going to have a great effect on growth and drug transformation.

1.4 Improvement of Yields

Final drug conversion rates and levels can be improved by three methods, the identification of optimal fermentation conditions, genetic manipulation and breeding.

The first of these methods was to be attempted during this project. The most useful experiments are the ones carried out in continuous culture as in theory they allow for the alteration of single factors whilst other factors remain constant. Batch work suffers from the disadvantage that cell density is always altering and the age distribution of the population also alters considerably. The theory of batch and continuous culture has been extensively discussed by Pirt and others (4,96). In many commercial fermentations the product is only produced at the end of the exponential phase (2,80) and therefore batch investigations may be the only method of studying product formation.

Chemostat studies have shown that cyt P-450 systems in Candida tropicalis can be expressed in continuous culture (47). It is therefore probable that other organisms may be capable of expression of cyt P-450 systems whilst growing in continuous culture. It was hoped that the organism chosen for the project would be

capable of growth in a chemostat.

1.5 The Choice of Organisms for the Investigation of N-dealkylation of Drug Intermediates by Fermentation

The possibility of carrying out N-dealkylations by using various heterotrophic bacteria and several filmentous fungi was investigated by Sewell (108). He showed that the required reactions were carried out by some of the fungi. As a result a preliminary investigation of N-dealkylation by a Cunninghamella strain was carried out in shake flask and other batch fermentations (1 and 7 liter stirred tank scale). The

The literature survey in section 1.3 suggests that several yeasts may also have the ability to carry out the bioconversions at present being performed by Cunninghamella strains. Preliminary work indicated that several strains of the yeast Candida tropicalis possess the ability to N-dealkylate codeine to norcodeine but conclusive results were not obtained (45). As most of the work was carried out using C.bainieri the decision was made to continue using this organism. Even though C.bainieri had been found to carry out N-dealkylation reactions previously (39,40), results obtained with Cunninghamella spp must always be treated with caution as the taxonomy of this group is uncertain (71). It was necessary to understand the properties of filamentous fungi before attempting to grow C.bainieri on a large scale.

1.6 The Growth of Filamentous Fungi in Submerged Culture

1.6.1 Introduction

Filamentous fungi exhibit a mycelial growth form which is ideal for growth on or through solid and porous media such as soil (14), and the Cunninghamellae are organisms found mainly in the soil (32,48,71). This growth form is far from ideal for use in fermenters designed for suspension cultures. In submerged culture these organisms may grow as mycelial filaments, pellets or attached mats and lumps. When attempting to scale up from shake flasks to larger fermenters the type of growth obtained is of great importance because it affects the type of fermenter used, sampling and assay techniques, growth rate etc. Different growth forms and how they occur are discussed. The effects and desirability of the different growth forms are also considered.

1.6.2 Mycelial growth

Fungi have been observed growing as individual mycelia in shake flasks and larger fermenters (8,9,16,34,43,59,64,73,87,97,99,100,103,122,124,125). Analysis of the growth kinetics of mycelial cultures has shown that the optimum growth rates can be either linear or exponential (16,74). Linear growth is caused by the growth of mycelial strands from one point (122) whereas the logarithmic relationship occurs when the mycelia branch regularly (16,18,122). Logarithmic kinetics are

also possible because of the homogenous distribution of mycelia in the medium. This enables all the mycelia to remain physiologically young and grow at their maximum rate (16,24). If the mycelia are repeatedly broken by shearing during the fermentation, it is possible to grow the organism in a chemostat culture which enables a very powerful tool to be used to study the fermentation of the organism.The model used to describe such cultures when limited by only one nutrient is the Monod model. This model is used extensively, both in its basic form and in modified forms to interpret the results of batch and continuous fermentations (4,96). Monod kinetics are described by the following equation-

Equation i

$$\mu=\mu_{max}((s)/K_m+(s))$$

where μ = specific growth rate

μ_{max} =maximum specific growth rate

(s)=limiting substrate concentration

K_m =the Monod constant

-the concentration of substrate at which $K_m=1/2\mu_{max}$

1.6.3 Pelleted Growth

Burkholder and Sinott (19) found that of 150 strains of fungus studied all formed pellets when grown in shake flasks. Pellets seem to be the major growth form of filamentous fungi in shake flask cultures. Large variations in texture, size and shape of pellets have been described and seem to be related to the age of

pellets, fungal strain and culture conditions (14,16,19,34,43,86,113,112-115,127,131,133).

Both the size and structure of a pellet can have effects on its growth kinetics. Small loose pellets may exhibit Monod kinetics (equation i) whereas large dense pellets will show a cube root relationship for growth as given in equation ii, equation,iii and figure 1.2 (122-125)

Equation ii

$$M^{1/3} = K_t + M_o^{1/3}$$

M = biomass at time t mg cm^{-3}

M_o = biomass at time zero mg cm^{-3}

K = constant - NOT specific growth rate.

Pirt (96) proposed that the growth of cultures containing a number of pellets could be described by Equation iii

$$M^{1/3} = (4\ \pi\ p\ n/3)^{1/3} awt + M_o^{1/3}$$

M = total masses

p = dry weight of organism per unit volume of pellet

w = Width of peripheral growth zone of the pellets

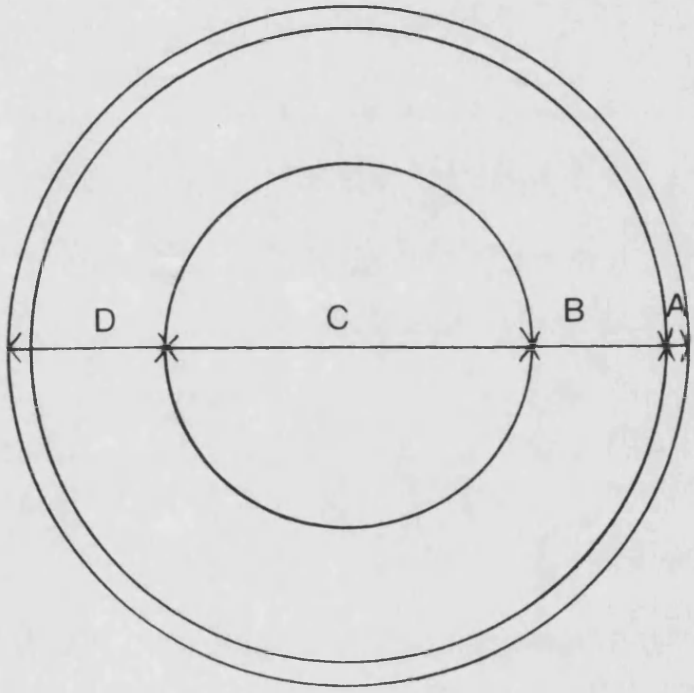
n = number of pellets in the culture

a = specific growth of the organisms

This was confirmed by the work of Trinci (123) who proposed the

Figure 1.2

The Proposed Zones of a Hypothetical Pellet of Aspergillus nidulans grown at 30 ^{o}C and 9 mm in Diameter (123).



A = Proportion of the growth zone (w) contributing to the radial growth of the pellet.

B = Proportion of the growth zone not contributing to the radial growth of the pellet.

C = Non growing zone 4 mm wide which is under anaerobic conditions. This zone autolyses slowly from the centre, producing inhibitors which diffuse outwards.

D = Growth Zone (g), 2.5 mm wide which grows exponentially at the maximum specific growth rate.

model for fungal pellets shown in figure 1.2.

Zoning is caused by diffusional limitations on nutrient uptake into the pellet. Pirt (96) suggested that the most probable limiting substrate was oxygen and that if it entered the pellets by simple diffusion growth of the pellet would be restricted to a 77μm wide peripheral zone. In real pellets the width of the growing peripheral zone may vary considerably because of changes in the density of the mycelia, bulk flow etc (16,61,123). Several examples will demonstrate the variability observed between pellets of different species of fungus grown under different conditions. The pellet shown in figure 1.2 possesses an unlimited growth zone 2.5mm wide. Clark (25), on the other hand, found that loose fluffy pellets of Aspergillus niger became hollow after reaching a diameter of 1.75mm. Increasing the oxygen tension in the medium led to smaller pellets forming and these pellets became hollow when they reached a diameter of only 1mm. This suggests that the pellets may have been denser and hence less easy for oxygen to penetrate, probably as a result of an increase in metabolic activity of the mycelia. Analysis of bacterial slimes did not show the same decrease in oxygen penetration when oxygen levels were raised (18).

The result of this diffusional limitation is that pellets become very heterogenous in structure. In some cases the centre of pellets can undergo autolysis and the pellets become hollow as a result of this limitation

(25). Consequently the physiology of the pellets is very complex, some pellets may sporulate internally (19), work on the effects of environment on the fungus is hard to interpret as a result of this heterogeneity of morphology and physiology.

1.6.4 Attached Growth

One of the most frequently used microbiological culture vessels is the petri dish. Colonies of microbes can be grown on the surface of agar contained in these plates. Growth of these colonies is sustained by the diffusion of nutrients from the agar and atmospheric oxygen from the air (in the case of aerobes). This type of growth often results in linear relationships for the increase in diameter of microbial colonies on agar surfaces (96,123). Attached microbial growth in fermenters may resemble the shape of cultures grown on agar.

In submerged culture the site of attachment of the fungus is unlikely to provide a source of nutrients as the surfaces are usually glass or stainless steel. Therefore the growth of the attached organism can be modelled as a section of pellet.

1.6.5 Pellet Formation

The literature on pellet formation has been summarised by two reviews (86,133). These reviews

suggested the possible modes of pellet formation listed below apart from mode iv:

i One pellet one spore - the spores germinate and each spore grows to produce one pellet.

ii Spore coagulation - the spores form groups which germinate and grow. only a few of the spores in the group would need to germinate to allow pellet formation.

iii Attachment of spores or filaments to solid particles in the medium and their subsequent growth could lead to pellet formation.

iv Detachment of entangled germinated spores from the surface of the growth vessel may also be a means of forming pellets (127).

v Agglomeration of hyphae - the hyphae become physically entangled in the body of the growth medium().

It is unlikely that a single mechanism is solely responsible for pellet formation in any culture. Reports of pellet formation by spore aggregation (43), mycelial aggregation (19) and pellet aggregation (123) have all been made.

Every aspect of the fermentation from spore age to pH affect the formation of fungal pellets and these factors often have very different effects on different strains of fungus (86). Some of the factors that were relevant to this work are as follows:

i Polymers

The presence of charged polymers may effect spore

agglomeration. Carbopol-934, Junlon 110 and Junlon 111 all of which are anionic polymers prevent the agglutination of Aspergillus niger spores and can result in either increased pellet production per spore (131) or dispersed mycelial growth (34,125). Other anionic polymers have similar effects on Aspergillus niger and cationic polymers have the reverse effect (34).

ii Surfactants

Surfactants have been shown to have very variable effects on pelleting. Span has been shown to decrease spore coagulation whilst tween increases coagulation and forms fluffy pellets with Aspergillus niger. These results were complicated by toxicity effects (125).

iii Spore concentration

Generally, high spore concentrations result in filamentous growth, Van Suijdam et al (131) quote typical values of less than 10^5 to 10^6 spores per millilitre for pelleting to occur in three fungal strains. Other workers report values of greater than $2x10^3$ spores per millilitre. No proportionality between spore concentration and pellet numbers was detected in work by Trinci (123) and this seems to be the case for most fungi, and is explained by an increased number of spores or mycelia being accumulated in the pellets.

iv Shear

High agitation rates and thus high shear in stirred tank reactors have been shown to be capable of changing pelleted growth to dispersed mycelial growth (86,97,131,133). Another effect of the high shear rate is the shortened thickened appearence of mycelial growth in these conditions (74). Consequently pellets grown at high shear rates tend to be smaller and denser than pellets grown at lower shear rates (86,133).

v Silicon Coating of Vessel Walls

Spores are often buoyant and tend to accumulate at the air/liquid interface in fermenters. They tend to be stranded at the extreme of the liquid levels in fermenters (127). In small culture vessels, especially shake flasks, the accretion of spores, mycelia and small pellets form a ring which can contain the majority of the biomass in the vessel(127). Ugalde and Pitt (127) found that siliconising the walls of shake flasks prevented much of this accumulation during the culture of Penicillium cyclopium. By reducing surface tension at the air/medium interface siliconisation of the flask wall eliminated a site where the aggregation of spores and tangling of hyphae during germination occurred. Consequently many more smaller pellets were formed than in non siliconised flasks, growth rates were doubled and

standard deviation between growth curves reduced.

1.6.6 Attached growth and its Initiation

The phenomenon of attached growth in fermenters is familiar. Unicelluar organisms may coat a fermenter with a layer one to several cells thick (4) and accumulate in large quantities at some points (4). It is harder for the more massive fungi to attach to glass walls that are constantly washed with medium, especially if the walls have been siliconised. Dead spots, unwashed surfaces, uneven surfaces and small gaps between surfaces all provide likely spots of attachment for fungal growth. This type of growth is not desirable in normal ALF or STR cultures as it makes growth analysis difficult, can impede the flow of medium, cause disruption of sampling devices and probes and may represent a large population of the growth in a small fermenter.

Rowley and Bull have recommended that vessels for the culture of dispersed fungal mycelia should be devoid of baffles, have few intrusions, be filled as much as possible to ensure that surfaces were washed with medium and that siliconisation be used on the vessel (106). They also recommended a minimum fermenter size of one liter to prevent clogging of the fermenter. Attached growth will be discussed in greater detail in the experimental results.

1.6.7 Summary of the Features of Fungal growth

a)General Features of Dispersed Mycelial Growth

Mycelial growth can often be induced by the use of large concentrations of spores as inocula (21,30,86,113,131). Growth is normally of the Monod type and the culture is comparitively homogenous. This type of growth is easy to use for biochemical and physiological studies because

i It is easy to take representative samples from this type of culture (103).

ii Continuous culture in chemostats may be possible (97,106).

iii Biomass can be assessed using light absorbance techniques, giving an almost instantaneous estimate (124,125).

b)General Features of Pellets

Pellets are normally formed when low concentrations of inoculum are used (21,131), the concentrations varying with strain and culture conditions. They tend to be smaller and denser at high agitation speeds (86,133). Agitation and other factors which prevent spores from aggregating or accumulating at certain points, will in general, produce larger numbers of pellets or even mycelial growth (86,97,131,133).

In many cases pellets are not a suitable growth form to use for basic biochemical or physiological work. This is especially true of larger pellets which exhibit

i Slower growth kinetics than filaments (122,123).

ii Less easy to use for continuous culture (97).

iii Representative samples are difficult to obtain from fermenters because of the size and distribution of pellets.

iv Growth inhibitors may be produced at the centre of large pellets (96).

v It is easy for pellets to become lodged at various points within the fermenter.

vi Diffusional limitations cause the pellets to be heterogenous in structure (123).

However, pelleted growth can be a great advantage for the following reasons:-

i Some processes such as the production of mushrooms in submerged culture depend on the death and autolysis of fungal biomass to provide important components which make the final product saleable (86,131). In this case flavour development is dependent on autolysis of the pellets.

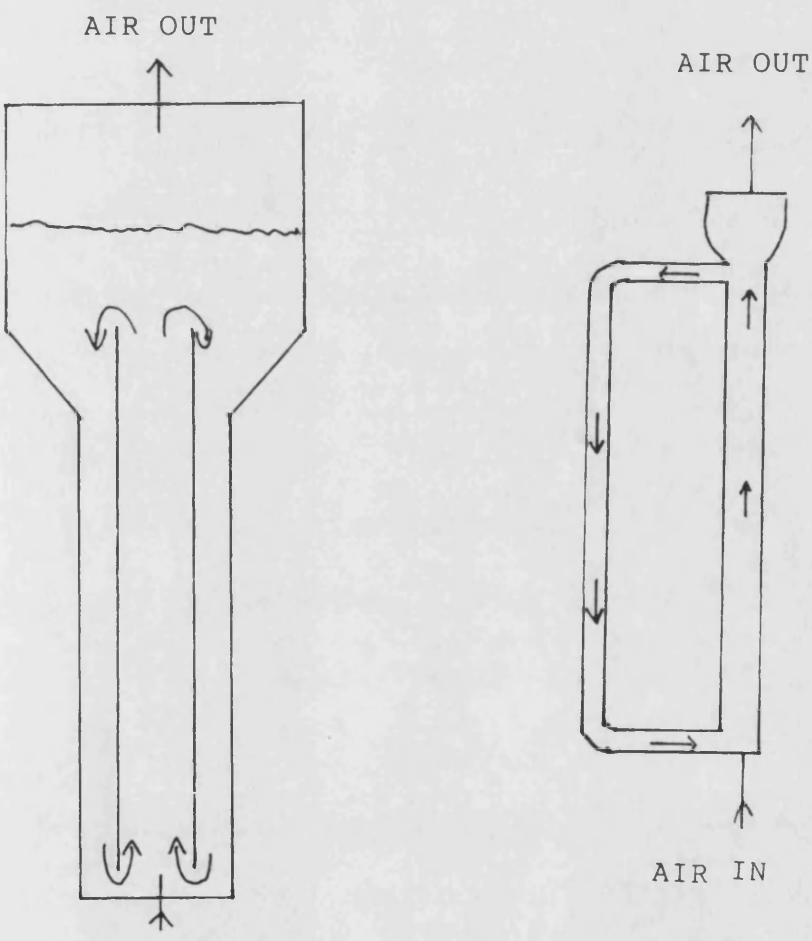
ii The reduced viscosity of most pelleted cultures compared to mycelial cultures aids mixing (5,26,36,59,87,136) and has been the growth form of choice in most ALFs (6,62).

iii Pellets are easy to remove by filtration, centrifugation and settling techniques.

iv Pelleted biomass can be retained in continuous fermenters relatively easily (63).

Figure 1.3

Internal and External Draught Tube Fermenters



INTERNAL DRAUGHT TUBE FERMENTER

EXTERNAL DRAUGHT TUBE FERMENTER

1.7 Scale up of the fermentation of Cunninghamella bainieri

1.7.1 Introduction

The process by which a laboratory scale fermentation is increased to pilot plant or industrial size to enable large scale growth and/or product formation is called scale-up.

The bioreactor system used for scale up should allow:-

1 Adequate aeration if the culture is aerobic and mixing of the growth medium

2 Control of culture parameters eg temperature, pH and foaming

3 Aseptic operation of bioreactor if necessary.

There are many bioreactor designs which can be used to achieve the above aims. SFTs, tubular fermenters, airlift fermenters, fluidised beds and fixed bed reactors can all be used (Atkinson and Mavituna, Bailey and Ollis) (4, 137). These reactors can also be run using a variety of regimes such as batch or contiuous culture (137). The regime and reactor should be chosen to suit the organism used for scale up.

The strain of organism used for a process must therefore be chosen with care (if any choice exists). The number of organisms which carried out N-dealkylation reactions of the type under study was extremely limited (108, 109).

1.7.2 Strain Selection

Organisms used for biotechnological purposes must:-

1 Grow under laboratory conditions

2 Carry out the desired reaction (eg antibiotic production (2,

29, 80), drug conversions (2, 58) or biomass production (73)). Screening for such organisms can be carried out using small scale cultures in test tubes, petri dishes and shake flasks (eg 2, 3, 109). A small number of the most promising isolates are then chosen for futher investigation and comparison. One of these may then be chosen for scale-up.

An ideal organism for exploitation would

1 Produce as much product per unit biomass as possible without killing the cells or preventing the growth of high densities of biomass.

2 High cell densities should be obtained, but not inhibiting product formation.

3 Once high cell densities are obtained productivity should remain high, cell viability should remain high but cell growth and division should be minimal.

4 The cells should be easy to grow (nutritionally undemanding and if grown in suspension, easy to mix - no excessive changes in viscosity, and hence power requirements).

5 Product should be present in a form which is easily recovered and purified.

6 These advantageous properties should be stable over time.

If product formation is limited to active cell growth and metabolism, then it is desirable to either maintain rapid cell growth and hence biomass production (the logarithmic growth phase (Section 1.6) or maintain the cells at the lowest growth rate that will enable efficient product formation. Other products are

formed near the end of a batch fermentation (stationary phase) where cells are dying and in nutritionally unbalanced conditions. N-dealkylation of C. bainieri is probably of this second type as it occurs only when glucose has been exhausted in the medium (Section 3).

The N-dealkylations carried out by C. bainieri should be best suited to culture regimes which allow the rapid growth of biomass and then the maintenance of productivity by the cells for as long as possible.

The choice of a filamentous organism for the N-dealkylation studies was necessary because no single celled organism which performed the N-dealkylations under investigation could be found (108, 109) . This was unfortunate becuase in terms of growth rate and control of cell physiology single celled organisms are preferable for use in submerged culture (96). Generally, cultures of single celled organisms are easy to handle, do not produce tough clumps of cells which autolyse or differentiate and have a much smaller effect on increasing medium viscosity than filamentous organisms (Sect 1.6).

Cultures of filamentous organisms can be difficult to mix and hence monitor and control as many form dense pellets which are impossible to assess physiologically (Sect 1.6).

Despite these difficulties, the experience of the pharmaceutical industry has shown that the large scale culture of filamentous microorganisms can be performed sucessfully eg penicillin (2). An advantage of filamentous cultures is that

biomass from the spent fermentation medium is easily recovered by filtration (4).

Shake flask experiments had shown that C. bainieri could be grown as filaments or pellets (Sect 3.3.6.3). This suggested that a wide range of fermentation strategies should be available for the growth of C. bainieri and no codeine production from the resultant biomass. It appeared that both ALFs and STFs should be adequate for the culture of C. bainieri.

1.7.3 Culture Strategies

Atkinson and Mavituna (137) summarise the features of biochemical process plant

1 The volumetric rates of reaction are low

2 Plant is large compared to comparable production scales in the chemical industry

3 Power consumption for mixing and aeration is high

4 Because of the presence of cells and low product concentrations separation costs are high

5 Capital costs of product recovery equipment are high

6 Most processes are run as batch fermentation.

Very large volumes of reactant may enable continuous processes to run eg sewage treatment and SCP production. It therefore follows that the strategies used to produce a biological product should:-

1 Achieve as high a cell density as possible whilst allowing as

high as efficiency of product formation as possible.

2 The biomass should be in suitable physiological state for product formation to occur.

3 Mixing should be adequate for the provision and pH control.

4 If whole cells are needed for the reactor the cells must not be damaged to such an extent that product formation is hindered. High impeller tip speeds have been implicated in cell death of Tetrahymena (138) and hyphal damage of fungi (128). Immobilisation techniques can also lead to cell death (4).

Cultures can be grown in a variety of vessels and the cells used may be free floating or fixed onto a support. The type of vessel used depends on the oxygen demand of the organism and the difficulty of mixing different cultures.

If the scale up of a culture to large scale is envisaged, care must be taken to ensure that full attention is paid to the physical parameters of the system.

Scale-up of fermenters can lead to substantial changes in environment. Height increases alter the solubility of oxygen in the medium by increasing the pressure at the bottom of the vessel (Sect 1.7). Scale-up of linear dimensions for a series of geometrically similar vessels results in a cubic relationship with volume. Increasing the linear dimensions by a factor of 10 is equivalent to a volumetric scale up of 1000. This in turn leads to problems of aeration, agitation, heat transfer and medium sterilisation.

Mixing and aeration can also be hindered by the increase

in viscosity which can occur because of high cell densities, growth as hyphae, numerous small pellets or the production of viscous materials (Sect 5.1). A lowering of the oxygen transfer capacity of the system is a serious problem because of the low solubility of oxygen. Oxygen transfer from the gaseous to liquid phases is considered to be one of the major limiting factors of aerobic bioreactor design. High densities of rapidly metabolising cells require large quantities of oxygen. The low solubility of oxygen in water means that the reservoir of oxygen in solution must be replaced rapidly. Such cultures should be grown in vessels such as the STR which are designed to enable high rates of oxygen transfer, compared to ALFs.

1.7.4 Culture Strategies

1.7.4.1 Batch and continuous culture

There are two extremes of submerged culture operations. At one end of the spectrum is batch culture. An organism is grown in a vessel for a defined period of time after which the medium and cells are harvested.

The other extreme is chemostat culture in which a homogenous suspension of dividing cells or filaments is grown (96). Each approach has its advantages.

Batch culture is short term and flexible. Equipment can easily be used for different organisms and hence the production of small quantities of several different compounds can be achieved using a single piece of equipment. The physiology of

the cells in batch culture is varying continually as is the medium. This makes accurate analysis of the relationship between culture conditions and cell physiology difficult.

Continuous culture is less flexible than batch and is not used for many commercial purposes. When continuous culture is used it tends to be in large volume processes. However, continuous culture is widely used to provide data for analysis of cell physiology.

This is because cells can be maintained in a steady state, the distribution of different cells in the medium is constant as are all the medium parameters (96). Thus, the effect of altering a single parameter can be determined whilst maintaining all other parameters at a constant and known level.

Immobilised cells tend to be operated in a semi continuous mode. Cells are not easily lost from their support material and once the intial biomass is grown the strategy is to use the cells as a catalyst. Emptying the vessel of growth medium (drain and fill) is an attractive strategy for reducing the time taken in growing the organism compared to the time taken for product formation. The conversion medium may be removed after a suitable period of time (one to several days) and be replaced with another batch of medium. It may be possible to "milk" an immobilised culture for a period of weeks. Other techniques may involve the addition of a continuous stream of reactant to, and removal of product from, a vessel of immobilised particles. Alternately, closely packed columns can be fed continuously with

reactant. The range of combinations and possiblities is endless.

1.7.5 Culture strategies

Fermenter type and Fungal morphology

The growth form of microorganisms can have an enormous effect on the efficiency of culture vessels. Where fungi are concerned the major effects are rheology and attached growth.

Small pellets (eg pellets >0.5-1.5 mm diameter), and filaments produce non Newtonian rheology if they are present in any quantity (Chap 5.1). This makes mixing and oxygen transfer difficult in any type of fermenter. The effect is particularly serious in airlift reactors (ref 5, 5.1). Consequently, filamentous fungal cultures are best carried out in STFs. Larger pellets can be grown in either ALFs or STFs and there appears to be little difference in the efficiency of growth of pellets in either fermenter (Chapter 6).

Attached growth is a feature of fungal cultures which is always present to some extent. It should be possible to reduce the amount of attached growth by eliminating dead spores and keeping the medium clear of obstructions. Items such as baffles should be an integral part of the fermenter wall to prevent the lodging of fungi between the vessel wall and the baffles. A build-up of attached growth can lead to the ending of fermentations by removing a large quantity of biomass from contact with the media. This is especially true in small batch scale fermenters (1 l) where the probes etc occupy a large volume compared to the medium. In ALFs, accumulations of biomass can

act as a valve and reduce the rate of liquid circulation, this in turn can lead to the settling of more material.

The use of large fermenters should reduce the effect of attached growth because of the relationship between linear dimensions and volume mentioned previously.

Attached growth will also give erronious results for continuous culture experiments. Organisms which prefer attached growth may be cultured on some sort of carrier other than attempting growth in suspension.

1.7.6 Reactors

This information is obtained from Atkinson and Mavituna, Bailey and Ollis, (4, 137).

1.7.6.1 Stirred tank fermenters

Widely used on laboratory and commercial scales and can be used in batch, fed batch and continuous modes. Single cells, pellets, filaments and cells immobilised on carriers can all be grown in STFs. Can achieve very high oxygen transfer rates.

1.7.6.2 Airlift reactors

Used for ICI SCP process and investigated for use with large particles and can be used for growth of single celled and filamentous organisms. Oxygen transfer rates in small models are not as good as in STFs of equivalent volume but adequate for slower growing organisms. Suggested advantages most apparent with increase in scale.

1.7.6.3 Packed bed

Medium is passed through a bed of material onto which microorganisms are attached.

This has the advantage that little biomass is suspended and growth of cells does not increase viscosity of the medium. Aeration, pH control etc take place in a separate tank. Medium can be recirculated through the bed or a large volume can be passed through the bed and continuously harvested. Organisms such as fungi which produce rapidly expanding spheres of biomass can soon block this sort of system.

1.7.6.4 Fluidised bed

This is similar to the packed bed but the immobilising support is light enough to be suspended by the upflow of medium through the bed, but dense enough to prevent washout of the particles. Fluid velocity within the vessel can be lowered by increasing the cross-sectional area of the vessel.

1.7.6.5 Plug flow

Medium pumped through the tubular reactor maintains flocs or pellets of cells in suspension at various heights up the reactor depending on the size and density of the flocs.

C. bainieri was known to grow in filamentous and pelleted forms in submerged culture in shake flasks. In view of the availability of equipment and background information available it

was decided to try and culture C. bainieri in free suspension in an airlift and on STFs (Chapter 5).

This gave the options of continuous, batch and intermediate cultures. One of the priorities of the work was to develop a greater understanding of the effects of culture parameters on N-dealkylation of drug intermediates by C. bainieri. Continuous culture was the most desirable strategy for such investigations. Armed with basic information from continuous runs interpretation of batch data from mycelial and pelleted cultures should be comparitively easy.

v Pellets may be useful anchors for immobilised enzymes (131).

1.8 The Airlift Fermenter

1.8.1 Introduction

Airlift fermenters are intended to provide adequate mass transfer, mixing and suspension for the growth of the cultured organism without the use of impellers. The act of sparging air into any liquid filled vessel will cause some mixing and the effect of sparging from a localised area will induce liquid circulation. This effect was observed in the STF during air sparging when the impeller was not rotating. Designs of fermenter which have been used to take advantage of the mixing effects of sparged air include bubble columns (24), external loop ALFs (10) and internal loop ALFs (10). Poor mixing and mass transfer in bubble columns compared with the two types of ALFs make bubble columns less attractive than ALFs as culture vessels (111).

The main difference between the two ALF types is the position of the risers and the downcomers (fig 1.3). Internal draught tube fermenters are normally more compact than the external loop design and easier to alter in the laboratory.

Simplicity of construction and operation has led to the use of ALFs in several laboratories. Tables 1.1

and 1.2 show the extent of variation between different working designs of ALFs and the range of organisms cultivated. Algal cells (48), yeast (52), bacteria as single cells (16), bacteria with particulate media(eg 56), immobilised cyanobacteria (91,92), bacteria immobilised on alginate(60), filamentous fungi as mycelia (8,9), filamentous fungi as pellets (6) and filamentous fungi with particulate matter (129) have all been grown using airlift fermenters. Sewage treatment (94) and surfactant production (77) have also been carried out using airlift fermenters.

There are several distinct advantages to using a system in which mixing is powered by the input of a vital reactant, in this case air:-

i The need for mechanical seals on rapidly rotating shafts is eliminated (10).

ii Elimination of mechanical agitation reduces the risk of interference caused by motor breakdown. There is still a risk of air compressors failing but air compressors are generally required for STFs anyway.

iii Modelling and hence scale up of airlift fermenters is much easier than for STFs (111).

iv There are few sites for the attachment of filamentous organisms.

v Airlift fermenters have been successfully used to study most types of growth (tables 1.1 and 1.2).

vi The tall narrow shape of fermenters enables efficient heat transfer to take place (90).

Table 1.1

Internal Recycle Air Lift Fermenters and their Uses

Volume	Other Information	References
?	Very primitive	70
200l	Modelling, growth of Candida intermedia	52
7.25l,100l - -	Comparing the growth of Aspergillus oryzae in two ALFs with an STF	6
850ml, 8.5l	Immobilised cyanobacteria	91,92
?	Continuous commercial alcohol production	38
30l	Growth of Methylophilus methylotrophus	41
4l	Comparison of ALF and STF	57
10l -	Growth of Candida utilis at different pressures	93
?	Sewage, 0.5 - 10m in diameter	94
15l	Modelling	32
3l - -	Filamentous fungi using a vibromix unit to achieve filamentous growth	64

Table 1.2

External Recycle Air Lift Fermenters and their Uses

Volume	Other Information	References
111	The effects of *Aspergillus niger* on heat transfer and rheology	8,9
-	Sampling mycelial cultures	103
-	Effect of gas inlet design	83
560ml	Removing pyritic sulphur from a 5% coal slurrey	56
-		
10ml	Continuous algal cultures	98
5l	Cellulose degradation by *Sporocytophaga myxococcoides*	129
-		
?	265 cm high and 15 cm diameter *Hansenula polymorpha*	15
-	modelling	19
?	Similar to the above ALF	-
-	Culture of *E.coli*	69
-	Process simulation of single cell protein production	72
-		
-	Continuous cell production	73
-	Modelling	84
-	Continuous penicillin production	62
?	Similar to the above ALF but with single and multi stages, used to grow *E.coli*	-
-		1,107

vii It is claimed that the use of air for mixing is much cheaper than stirring with an impeller and hence operating costs are greatly reduced (10).

viii Effective foam suppression can be caused by suitable direction of the circulation flow (10).

ix A large gas liquid interface is provided by this method (10,90).

x Airlift fermenters have been shown to provide a low shear environment which is ideal for the growth of sensitive cells (56,64).

Several disadvantages of the airlift system have been mentioned:-

i Oxygen transfer rates tend to be lower than in comparative STFs (6).

ii Economies are difficult to achieve regarding power input because air compression is inherently inefficient (66).

iii In tall vessels stripping of oxygen from the medium by depleted medium may occur(66).

iv As the hydrostatic head is increased air compression power must also increase(66).

The use of large airlift reactors for sewage treatment (94) and ethanol production(33) has demonstrated that there is a potential market for ALFs. It is also noted that in larger fermenters (94) air does not need to be injected into the bottom of the fermenter thus reducing the compression power needed. Larger fermenters also have the advantage that the air bubbles

injected into the downcomer are totally dissolved near the bottom of the fermenter due to the pressure of the liquid column and problems of gas transfer across a gas liquid interface are eliminated.

In conclusion it seems likely that airlift fermenters will be of greatest use in areas where the fermentation medium is of low viscosity, oxygen demands are moderate, and the biomass circulating is sensitive to shear. Fungal pellets and gel immobilised cells are obvious candidates for this system.

1.8.2 Airlift Theory

The basic theory of the airlift pump is well understood and can be found in basic texts such as Coulson and Richardson Volume 1 (28). The theory shows that in a system in which there was no frictional resistance the mass of air required to pump a unit mass of liquid (m/M) would be equal to

$$\frac{h_r\, g}{P_a\, v_a \ln((h_s + h_a) / h_a)}$$

where

g = acceleration due to gravity

$h_a = h_r + h_s$

h_r = The height of the liquid in the rising main above the reservoir surface.

h_s = The height of the surface of the liquid reservoir above the air inlet.

P_a = atmospheric pressure

v_a = specific volume of air at atmospheric pressure.

Apart from the frictional losses experienced in all airlift pumps other inefficiencies are introduced in airlift fermenters. One of these is caused because the systems are closed loops. The headspace is seldom totally degassed and this results in considerable gas holdup in the downcomer at higher gas flow rates and consequent lowering of circulation rates (52). The rheology of the medium also has a considerable effect on the efficiency of airlift fermenters (62).

1.9 A Brief Introduction to ALF and STF Experiments

It was already known that ALFs could be sucessfully used to grow pelleted forms of filamentous fungi (tables 1.1 and 1.2). The ALF was constructed for the purpose of cultivating C.bainieri with the hope of using the fungus to N-dealkylate codeine within the fermenter. Alteration to the design of the spargers and draught tube were carried out during the project but the medium composition was kept constant. Different inoculum techniques, PTFE spraying and the effect of an antifoam were also examined in these experiments.

Most large scale aseptic culture of microorganisms is carried out in STFs. Books such as Pirt (96) and Bailey and Ollis (4) cover the basics of STF culture in detail and no attempt to repeat the basics will be made here.

The fermenter used was an LH series 2000 model which is of a type primarily designed for the culture of

single celled organisms. In order for such a fermenter to be used to grow fungi several modifications normally have to be made (106). These include the removal of baffles and coating the vessel with PTFE.

Attempts were made to study the effects of stirrer speed, PTFE coating, the presence of baffles and intermittent air sparging on the growth of C.bainieri.

CHAPTER 2

MATERIALS AND METHODS

General

This section documents materials and methods which were used in more than one section of work.

2.1 Materials

2.1.1 Organism and Media

Cunninghamella bainieri C43 was obtained from the American Cyanamid culture collection. Inorganic growth medium constituents, anhydrous glucose and EDTA were all of analytical grade. Water was single distilled. Antifoams B and C were supplied by Sigma. Acid casein hydrolysate, malt extract agar, malt extract broth, tryptone soy broth and tryptone soy agar were all obtained from Oxoid limited.

2.1.2 Glucose assays

The glucose assay enzymes and buffers were obtained ready prepared from Sigma as glucose kit No 510-A. A Cecil U.V. scanning spectrophotometer model CE 588 was used to measure the absorbance of the samples held in plastic 1cm light path cuvettes. A Gallenkamp Tempette heater/stirrer and water bath were used. The automatic pipette was supplied by Anachem.

2.1.3 HPLC

HPLC grade solvents were used at all times and water was double distilled. Ammonium acetate was reagent grade.

The column packing material was obtained from Whatman, the Cecil CE 588 was used for much of the peak detection. Helium was obtained from BOC. Filters were obtained from millipore.

2.1.4 Codeine and Norcodeine

Codeine was provided by the School of Pharmacy of the University of Bath. Norcodeine was produced by chemically N-dealkylating codeine. Analytical grade reagents were used to N-dealkylate codeine and purify the norcodeine which was produced. All reagents except 2,2,2 trichloroethylchloroformate (2,2,2 TCEC) were obtained from BDH. Sigma supplied the 2,2,2 TCEC.

2.1.5 Miscellaneous

a) Filters for filter sterilisation were obtained from Millipore.

b) Filters for dry weight estimations were obtained from Whatman.

c) The counting slide was supplied by Gallenkamp.

d) Unless otherwise stated sterilisation of materials ws carried out using steam at 121^{o}C and 15 psig for 20 min.

e) Erlenmeyer shake flasks were obtained from Gallenkamp.

f) A Swift Phasemaster microscope was used for all microscope work.

2.2 METHODS

Organism and Media

Subculturing was carried out once every two weeks. Malt extract agar slopes were used to grow the stock cultures. Originally the cultures were grown for 2 days and then stored at $4^{o}C$. At later stages in the work the fungus as grown for 10 days to ensure heavy sporulation before storage.

Basic information about the culture media used is given in tables 2.1 to 2.5. Prepared media from Oxoid was made up and sterilised according to the instructions on the packets.

Filter sterilisation was carried out using 0.22 um membrane filters. Small volumes of glucose solutions and amino acid mixtures were sterilised using this method. Therefore, 2.5 cm diameter filters held in swinnex filter holders were sufficient to carry out the sterilisation. Syringes were used to pump the media through these filters into sterile containers.

CHAPTER 2

Tables 2.1 to 2.5

Media used for the Culture of C.bainieri in Shake Flasks and Fermenters

Table 2.1

GLUCOSE SOLUTION FOR ROBINSONS AND ELJ MEDIA

A solution of 100 g/l of glucose in sterile distilled water was made and filter sterilised.

AMINO ACID SOLUTIONS FOR ROBINSONS AND ELJ MEDIA

A 30 g/l solution of amino acid mixture was made in sterile distilled water and filter sterilised.

PRIMARY CULTURE

Component	Volume per flask (ml)
Robinsons double strength	25.0
Glucose Solution	5.0
Amino Acid Solution	5.0
Sterile Water	15.0

Inoculate from agar slope or using spores and incubate at 27°C and 250 rpm for 2 days.

SECONDARY CULTURE

Component	Volume Per Flask mg/ml
ELJ 1	25.0
ELJ 2	0.5
Glucose Solution	5.0
Amino Acids	5.0
Sterile Water	14.5

Inoculated with 10 ml of primary culture and incubated for 10 days at 27°C and 250 rpm.

20 mg of codeine phosphate per flask was added after one day.

Table 2.2

AMINO ACID MIXTURES

AMINO ACID	WEIGHT (grams)
Arginine	2.0
Aspartic Acid	5.0
Glycine	1.0
Isoleucine	4.0
Leucine	5.0
Lysine	5.0
Methionine	1.0
Phenyl Alanine	2.0
Threonine	2.0
Tyrosine	1.0
Valine	4.0

Table 2.3

ROBINSONS MEDIUM - MINERAL SALTS

TO MAKE 5 LITRES OF MEDIUM

MINERAL SALT	WEIGHT (grams)
KH_2PO_4	3.78
$NaH_2PO_4.2H_2O$	9.89
$(NH_4)_2SO_4$	6.67
Na_2EDTA	0.6670
$MgSO_4.7H_2O$	0.2800
$CaCl_2.2H_2$	0.0220
$FeSO_4.7H_2O$	0.111
$ZnSO_4.7H_2O$	0.2200
$MnSO_4.4H_2O$	0.0220
$CuSO_4.5H_2O$	0.0083
Na_2SO_4O	0.5600
$Na_2MoO_4O_2.H_2O$	0.0560

Add in the order given, adjust to pH 6.5 and autoclave at $121^{o}C$ for 20 minutes.

Table 2.4MODIFIED ELJ MEDIUM - MINERAL SALTS

TO MAKE 2 LITRES OF MEDIUM

Part 1

K_2HPO_4	5.440g
$NaH_2PO_4.2H_2O$	21.48g
$(NH_4)_2SO_4$	2.000 g
Na_2EDTA	0.808 g
$MgSO_4.7H_2O$	1.160 g

Add in the order given, adjust to pH 6.5 and autoclave at 121°C for 20 minutes

Part 2

A/

$CaCl_2.2H_2$	0.33 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.93 g
$FeSO_4.7H_2O$	0.035 g
$ZnSO_4.7H_2O$	0.010 g

Make up to 80 ml with sterile distilled water. Add 10 ml from section B and make up to 100 ml with sterile water. Filter sterilise.

B/

$MnSO_4.4H_2O$	0.010 g
$CuSO_4.5H_2O$	0.010 g
$Co(NO_3)_2.6H_2O$	0.0125 g
$Na_2B_4O_7.H_2O$	0.009g

Make up to 100 ml with sterile water and add 10 ml to section A.

Table 2.5
THE CULTURE MEDIUM USED FOR THE CULTIVATION OF CUNNINGHAMELLA SPP IN LARGE FERMENTERS

TO MAKE 5 LITRES OF MEDIUM

CARBON SOURCE

Glucose	50 g

NITROGEN AND CARBON SOURCE

Acid casein hydrolysate	15g

NUTRIENT SALTS

KH_2PO_4	6.8g
$NaH_2PO_4.2H_2O$	26.85g
$(NH_4)_2SO_4$	2.5g
Na_2 EDTA	1.01 g
$MgSO_4.7H_2O$	1.45 g
$MgSO_4.7H_2O$	1.45 g
$CaCl_2.2H_2$	0.527 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.775 g
$FeSO_4.7H_2O$	0.029 g
$ZnSO_4.7H_2O$	0.0083 g
$MnSO_4.4H_2O$	0.0082 g
$CuSO_4.5H_2O$	0.0083 g
$Co(NO_3)_2.6H_2O$	0.0104 g
$Na_2B_4O_7.H_2O$	0.007g

2.2.2 Glucose Assays

2.2.2.1 Method

The method used by Sewell (108) was used. The following reactions took place:-

GLUCOSE + H_2O + O_2 — GLUCOSE OXIDASE → GLUCONATE + H_2O_2

H_2O_2 + O-DIANISIDINE — PEROXIDASE → OXIDISED O-DIANISIDINE (BROWN)

Care was taken when handling the glucose assay solutions as O- dianisidine is cumulative poison, potential carcinogen and absorbed by the skin. The reagents were obtained as capsules labelled PGO enzymes and a bottle of O-dianisidine di hydrochloride.

One PGO enzyme capsule was dissolved in each 100ml volume of water in a foil covered bottle (reagent A).

The O-dianisidine di hydrochloride was reconstituted by adding 20ml of water to the bottle in a fume cupboard (reagent B).

One point six ml of reagent B was added to every 100ml of reagent A (reagent C).

Reagent C was stored for a maximum of 14 days at $4^{o}C$.

2.2.2.2 Glucose Standard Curve Determination

A series of standard solutions were prepared ranging from 0.5 to 3.0 mg ml^{-1} of glucose in medium plus 1 mM codeine phosphate. Each standard was diluted 20 fold with distilled water and a 0.5 ml aliquot pipetted into a

stoppered test tube. Five ml of Reagent C were added to the pre warmed test tube and mixed for 10 sec with a whirly mixer. The tubes were then incubated in a foil covered water bath at 37°C for 30 min. The samples were then cooled to 20°C in a foil covered water bath and the absorbance at 450nm measured using a zero glucose sample as a blank. The curve was found to be a straight line (appendix 1). An automatic pipette was used to carry out the dilutions as it had a range of 0.5 to 5.0 ml and speeded up the assays considerably.

Samples were diluted 100, 20 and 10 fold depending on the stage of the fermentation and then assayed using the methods described for standard curve determination. Standard glucose solutions were always used.

2.2.2.3 The Effects of Antifoam on Glucose Assays

Medium originally containing 100ppm of antifoam B, antifoam C and control medium were all compared. A final concentration of 0.1mg ml^{-1} of glucose was achieved by diluting the samples by 1/20. The results are shown in table 2.6. There was no detectable effect of either antifoam on the assay.

2.2.2.4 Assay Reproducibility

Fifteen samples of 0.1 mg ml^{-1} standard solution were assayed to check the reproducibility of the assay

TABLE 2.6
A TABLE TO SHOW THE EFFECTS OF ANTIFOAMS B AND C ON THE GLUCOSE ASSAY

SAMPLE -	A 450 REPLICATES - 1	2	3	4	MEAN
0.1 mg ml^{-1} GLUCOSE	0.27	0.27	0.27	0.27	0.27
GLUCOSE PLUS ANTIFOAM B	0.27	0.27	0.27	0.28	0.27
GLUCOSE PLUS ANTIFOAM C	0.26	0.27	0.27	0.27	0.27

TABLE 2.7

THE ASSAY REPRODUCIBILITY TEST, A_{450} FOR 15 0.1 mg ml^{-1} STANDARD SOLUTIONS

0.295	0.295	0.290	0.275	0.290
0.280	0.290	0.280	0.290	0.285
0.280	0.290	0.290	0.295	0.290

MEAN = 0.29
STANDARD DEVIATION = 0.06

technique. Table 2.7 shows that the results had a standard deviation of only 0.06.

2.2.2.5 Stability of Reading After Cooling to 20°C

A 1/20 dilution of a 1mg ml^{-1} glucose standard was assayed in triplicate. The samples were placed in a foil covered water bath at 20°C after 30 min. After 5,15,30,45 and 60 minutes the absorbances of the samples at 450nm (A_{450}) were taken. The variation of the replicates shown in table 2.8 is sufficiently low to allow the samples from assays to be left for at least an hour at 20°C.

TABLE 2.8

THE VARIATIONS BETWEEN A_{450} READINGS OF GLUCOSE ASSAY SAMPLES AFTER COOLING TO 20°C

TIME(MIN)	A_{450} OF SAMPLES		
-	SAMPLE 1	SAMPLE 2	SAMPLE 3
5	0.24	0.24	0.24
15	0.25	0.23	0.26
30	0.25	0.25	0.25
45	0.24	0.24	0.25
60	0.24	0.24	0.24

2.2.3 HPLC

2.2.3.1 Column Materials

The HPLC column was a 15 cm long, 0.5 cm i.d. stainless steel tube packed with partisil 10 ODS II reverse phase spheres.

2.2.3.2 Column Packing

The column with frit and bottom fittings attached was cleaned by filling with concentrated nitric acid and leaving at room temperature for 15 min. It was then emptied and rinsed with double distilled water and isopropyl alcohol.

A slurry of packing material was made by carefully mixing 2.6g of partisil 10 ODS II with 60 ml of isopropyl alcohol in a fume cupboard. The column was then packed with the slurry until completely full and the top of the column was levelled.

A stainless steel frit and other fittings were fastened to the top end of the tube.

The column was attached to a 70 ml bomb and the contents packed with isopropyl alcohol at 5800 atm. Several 30 ml aliquots were passed down the column and the time taken for each of the aliquots compared with the last (table 2.9). After the first two aliquots the time taken stabilised and the second part of the packing procedure started.

Isopropyl alcohol at 5000 atm was used to slam

the column with 4x30 ml of liquid (table 2.10).

TABLE 2.9

RESULTS FOR THE FIRST PACKING OF THE HPLC COLUMN

TIME TAKEN FOR EACH 30 ML OF LIQUID TO FLOW THROUGH THE COLUMN AT 5800 ATM (min)	TOTAL TIME (min and sec) -	TOTAL VOLUME OF LIQUID PASSED EACH TIME (ml)
0-00	0.00	0
4-56	4-56	30
5-04	11-00	60
5-58	16-58	90
6-36	23-04	120
6-13	29-17	150
6-20	35-37	180
6-05	41-42	210
6-58	48-00	240

TABLE 2.10

THE TIME TAKEN FOR 30 ml ALIQUOTS OF ISOPROPYL ALCOHOL TO PASS THROUGH THE HPLC COLUMN DURING SLAMMING AT 5000 ATMOSPHERES

TIME (min and sec)	6-55	7-25	7-26	7-16
TOTAL VOLUME OF LIQUID PASSED(ml)	30	60	90	120

2.2.3.3 Mobile Phase

A water/methanol mobile phase was prepared for use by the following method:-

For 100 ml of mobile phase, dissolve 1.5 g of ammonium acetate in 60 ml of double distilled water.

Bring the solution to pH 5.3 with glacial acetic acid and then make up to 70 ml using double distilled water.

Thirty ml of HPLC grade methanol was added to the acetate solution and mixed.

A 4.5 µm membrane filter was used to filter the mobile phase. The mobile phase was then degassed by bubbling with helium for approximately 5 min, or exposing to a vacuum for several minutes.

2.2.3.4 Sample Treatment and Running Conditions

A 4.5 um pore size membrane filter was used to clean 1 or 2 ml volumes of sample which were then injected into a 100 µl sample loop.

The sample was then flushed from the sample loop into the column. Absorbance at 240 nm was measured and the peak height of the trace was used to measure codeine and norcodeine concentrations. Known concentrations of codeine and norcodeine were injected at the beginning and end of the runs to act as external standards.

The system was allowed to stabilise for one hour before beginning the assays.

2.2.3.5 Column Maintainence

After heavy use the top few millimetres of the column packing were removed and replaced with a packing of partisil and methanol slurry.

2.2.3.6 Codeine and Norcodeine Standards

Standards were made up by weighing at least 20 mg of codeine and norcodeine into 10ml volumetric flasks.The standards were then dissolved in methanol and the volume made up to 10 ml. Standard solutions for HPLC use were produced by diluting the stock solution.

2.2.3.7 Codeine and Norcodeine Calibration Curves

Calibration curves were constructed using the mean of two replicate readings for concentrations between 0.005 and 0.014mg ml^{-1} of codeine and norcodeine (appendix 2);

2.2.3.8 HPLC Reproducibility

A sample of codeine and norcodeine was assayed 8 times. The distance from the time of injection and the peak heights were measured. Differences in peak height and spacing of 1mm were common (table 2.11)

2.2.3.9 The Effects of Antifoams B and C and medium on the HPLC Assay of Norcodeine

Mixtures of medium containing norcodeine, norcodeine and antifoam B and norcodeine and antifoam C were assayed.

TABLE 2.11

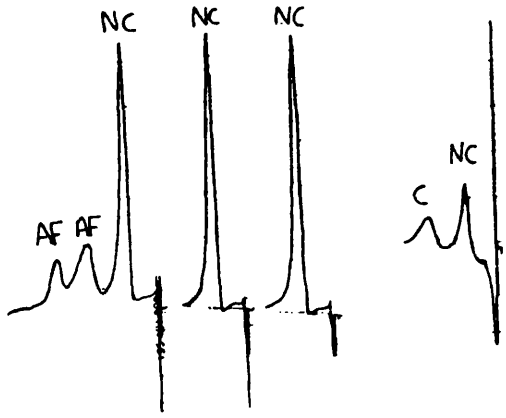
THE REPRODUCIBILITY OF CODEINE AND NORCODEINE HPLC ASSAYS, USING A CHART RECORDER SPEED OF 12 cm/hour

-	PEAK HEIGHTS(mm)		DISTANCE OF PEAK FROM POINT OF INJECTION (mm)	
-	CODEINE	NORCODEINE	CODEINE	NORCODEINE
-	7	18	21	10
-	8	18	21	10
-	7	17	21	11
-	8	16	22	11
-	8	16	21	10
-	8	17	21	10
-	7	17	21	11
-	8	16	21	10
MEAN	7.6	16.8	21.1	10.4
STANDARD DEVIATION	0.5175	0.7071	0.3536	0.5175

FIGURE 2.3

HPLC TRACES OF CODEINE AND NORCODEINE STANDARDS IN THE PRESENCE AND ABSENCE OF 100 ppm OF ANTIFOAM B

C = Codeine
NC = Norcodeine
AF = Antifoam Peak



The results were compared with the results for norcodeine in an ethanol standard. Figure 2.3 and table 2.12 show that the antifoam B has no effect on norcodeine detection.

TABLE 2.12

THE EFFECTS OF ANTIFOAMS B AND C ON CODEINE AND NORCODEINE HPLC ASSAYS, USING A CHART RECORDER SPEED OF 12 cm/hour

	NORCODEINE	NORCODEINE +ANTIFOAM B		NORCODEINE + ANTIFOAM C	
PEAK	75	75	18	15	73 14
HEIGHTS	76				
(mm)	75	75	18	15	73 14
PEAK	10	10	21	29	8 10
DISTANCES	10				
(mm)	10	10	21	29	8 10

2.2.4 The Conversion of Codeine to Norcodeine Using 2,2,2 TCEC

2.2.4.1 Outline of the Procedure

The codeine was first basified with sodium hydroxide. It was then esterified with pure dry 2,2,2 TCEC in dry solvents. The ester had then to be purified and converted to norcodeine. The norcodeine was then purified.

2.2.4.2 Basification

Twenty four grams of codeine phosphate was basified using approximately 15 ml of 5N sodium hydroxide. Water was then added until almost all the codeine was dissolved. The solution was divided into two equal volumes. Each volume was extracted with 3x 100 ml of dichloroethane in a 500 ml separating funnel. After the final rinse the aqueous phase was clear. An excess of anhydrous magnesium sulphate was used to dry the dichloroethane which was then filtered and evaporated to give the codeine base.

2.2.4.3 Preparation of the Chloroformate Ester

The codeine base was dissolved in 150 ml of toluene. Ten grams of dry sodium carbonate powder were heated fiercely for 10-15 min to drive off water, cooled and then added to the toluene. This mixture was placed in the apparatus shown in figure.

In order to ensure that the 2,2,2-TCEC was pure and dry it was distilled at 171-2^{o}C. Thirty millilitres of pure, dry 2,2,2-TCEC were dissolved in 60ml of dry toluene, placed in the 100ml separating funnel and stoppered. The reaction was started by slowly dripping the 2,2,2 TCEC into the codeine solution. When all the 2,2,2,-TCEC was added the mixture was refluxed for 5 days.

At the end of the 5 day period the mixture was

allowed to cool. Two hundred millilitres of diethyl ether were added to the toluene. The mixture was then decanted into a 1l separating funnel, leaving as much of the solid sodium carbonate as possible behind. Two 100ml aliquots of ether were used to wash the flasks to try and remove as much codeine ester as possible. The extract was pooled and then halved. Each half of the extract was washed with 2x 50ml of 2N hydrochloric acid and 50 ml of water. The toluene/ether mixture was then dried with magnesium sulphate, filtered and evaporated down at 60 to 70^{o}C to drive off the toluene and ether. A slight vacuum and gentle heating were used to drive off the 2,2,2-TCEC leaving a yellow oil.

2.2.4.4 Norcodeine Formation

The yellow oil was dissolved in 500 ml of glacial acetic acid; 36g of zinc dust added and the mixture was refluxed with stirring for 1 hour. Stirring was continued for 4 hours after the heating was stopped.

A bath of iced water was used to keep the flask cool while the contents were basified with sodium hydroxide pellets. Six 100ml aliquots of chloroform were used to extract norcodeine base from the resulting grey sludge. Gentle agitation had to be used to prevent the formation of an emulsion.

Anhydrous magnesium sulphate was used to dry the chloroform which was then filtered and evaporated down to give dark brown crystals which were dried under vacuum.

2.2.4.5 Norcodeine Purification

Acetone was used to dissolve the norcodeine base and then crystals were precipitated from the acetone using slight heat and vacuum. The filtrate from this process was recrystallised again. Both samples were pooled and redissolved, left at $4^{o}C$ to crystallise for 3 days, filtered and left for a futher three days to precipitate more norcodeine crystals. After vacuum drying the crystals were tested using NMR, HPLC and melting point determination.

2.2.4.6 Results for the N-dealkylation of Codeine Using 2,2,2-TCEC

1 Basification

Starting Weight - 24g

Theoretical Maximum Weight of Codeine base- 17.7g

Actual weight of codeine base - 16.6g

Percentage conversion - 94%

2 Norcodeine Base

Eight point two grams of impure norcodeine base was recovered from a grey/silver coloured slurry.

i) First Recrystallisation

Two and 2.1g of nearly pure norcodeine base were recovered and pooled.

ii) Second Recrystallisation

The pooled norcodeine base was recrystallised

twice. The first recrystallisation produced 2.42g of white needle like crystals, these were up to 3 cm long and had a slight brown tinge. One gram of slightly browner shorter crystals was precipitated from the residue.

Both sets of crystals were compared with norcodeine produced by Gibson (44) and published data. Melting point determinations gave values of between 183 and 185^{o}C compared with 185^{o}C given in the Merck Index (85).

Samples injected into the HPLC system all gave peaks at the same point. This was 11 mm from the point marking injection time on the chart recorder.

2.2.5 Spore Counting

An improved Nebauer Counting slide was used to count spores. The cover slip was pressed down firmly until Newtons rings could be seen. Both the slide and cover slip had to be alcohol cleaned before this phenomenon was observed. A small drop of water was placed on either side of the counting area to aid adhesion of the slide. Drops of media were then added to the counting chamber from each side until the chamber was full but not overflowing. Each side of the chamber was etched with a grid of 9 squares. The number of spores in the four outer squares and the central square of both sides of the chamber were counted. the mean number of spores was calculated and then multiplied by 10^{3} to give

the concentration of spores/ml.

2.2.6 Air flowmeter Calibration

A Lange Flowmeter was used to calibrate the gapmeters which were used to control the flow of air to the fermenters. The flowrate was either calculated by using data from at least three short calibration runs or from single 5 or 10 minute continuous runs.

2.2.7 Photography

Photographs of the fermenters were taken using a Minolta model SR-T 101 SLR camera. Two 150W photofloods were distanced 15 to 30 cm from the fermenter to provide illumination. A large sheet of non reflective black paper was placed behind the fermenters to provide contrast. A polarising filter was used to cut down reflected glare. The camera was placed on a tripod for all photographs and a cable release used to prevent blurring caused by handshake. Ilford XP1 400 film was used.

2.2.8 Shake Flask Experiments

2.2.8.1 Materials

a) the Kipps burette was supplied by Quickfit.
b) Tween 80 was obtained from Sigma.

2.2.8.2 Methods

a) Inoculum Techniques

Method 1

Fifty ml of Robinsons medium in a 250 ml Erlenmeyer flask was inoculated with a piece of mycelial mat from a 2 day old slope of C.bainieri. The flask was grown at 27°C and shaken at 250 rpm in a shaking incubator for 2 days. Ten ml of the culture were then used to inoculate 50ml of ELJ medium using a Kipps burette.

Method 2

Spores were added in varying volumes of distilled water (0.5 to 2ml) to flasks of growth media.

b)Shake Flask Culture Technique

Shake flasks were grown in a shaking incubator at 27°C and 250 rpm.

Dry Cell Weight Estimation

Whatman ashless filters were pre dried to a constant weight at 105°C and then weighed on a four figure balance. The filters were then used to filter the shake flask contents under gentle suction, rinsed and then dried to a constant weight at 105°C. The samples were cooled in a dessicator and weighed.

CHAPTER 3

Preliminary investigations

3.1 Introduction

Before pilot scale fermenter studies can be carried out on a microorganism, various exploratory experiments must be performed. These include experiments to optimise the medium, growth conditions and inoculum procedure using shake flask and other small scale fermenter vessels. Sewell(97) carried out experiments to determine the amino acid requirements, mineral salt requirements and the pH range necessary for the growth of Cunninghamella spp and conversion of codeine to norcodeine.

The work described in this chapter was carried out to complete the investigations necessary to enable the use of a 6 litre nominal volume ALF and a 12 litre nominal volume STF. Methods of inoculation, spore release, the effect of spore concentration on culture growth rate and morphology and the effect of antifoam on codeine conversion and uptake by Cunninghamella all had to be studied.

The methods, results and discussion of these experiments are presented in this chapter.

3.2 Spore Production and Release Experiments

Inocula for small volumes of medium were produced relatively easily by scraping spores from the surface of mycelial mats grown in small containers such as 1 oz

bottles or petri dishes. The possibility of producing larger concentrations of spores for use with larger volumes of media was investigated.

The experiments were divided into assessments of spore release, spore production, spore concentration and spore germination achieved by different methods. To prevent confusion the methods, results and discussion of each experiment will be presented under a single title.

3.2.1 Spore Release

3.2.1.1 The Effect of Tween 80 and Glass Beads

Method

When producing inocula for small volumes of media it was noted that the aerial mycelia of sporulating C.bainieri collapsed forming a thick layer which was difficult to remove spores from. A layer of air was also observed to be trapped beneath this layer.

The surface active agent Tween 80 was used to try and increase the wettability of the mycelia and facilitate the removal of spores by preventing the formation of the layer of aerial mycelia. Glass beads were used to mechanically agitate the mycelia with the aim of enhancing spore release.

Ten day old cultures of heavily sporulating C.bainieri were used unless otherwise stated.

Spore release was affected by the addition of 5ml aliquots of sterile liquid to fungal slopes in loz glass bottles.

The liquids used were sterile distilled water and a 0.1% v/v sterile solution of tween 80 in distilled water. Half the bottles contained glass beads (1.5 to 2.0mm in diameter) to a level halfway up the bottle. The bottles containing the beads were shaken backwards and forwards ten times over a distance of 15 cm and then whirly mixed at top speed for 10 seconds. Slopes in bottles not containing beads were scraped repeatedly with sterile pipette tips.

Eight universal 1 oz bottles containing sporulating mycelia were used. Two bottles were each given one of the following treatments. Spore removal using sterile distilled water and scraping with a sterile pipette tip, spore removal using sterile tween 80 solution and scraping with a sterile pipette tip and spore removal by shaking with a glass beads and water and tween 80 solution (table 3.1)

Table 3.1

THE NUMBER OF SPORES PER MILLILITRE OF LIQUID RELEASED BY TREATMENT OF SPORES OF SPORULATING C.BAINIERI WITH COMBINATIONS OF WATER, TWEEN 80 SOLUTION AND GLASS BEADS.

	+beads	-beads
+tween	4.6×10^6	2.6×10^6
-tween	3.9×10^6	1.4×10^6

CHAPTER 3

Discussion

The Effect of Tween 80 and Glass Beads

The data presented in the results section shows that both tween 80 and Ballotini beads had a beneficial effect on spore release and that a combination of the two more than doubled the concentration of spores released from mycelia. No significant visible increase in damage to the spores was observed after using any of these treatments. The presence of beads enhanced the release of spores much more than tween 80. Unfortunately the agar on which the spores were grown became mashed when shaken with Ballotini beads. This was difficult to remove from suspension and consequently the spore suspension was difficult to handle.

3.2.1.2 Spore Extraction by Shaking Mycelial Mats With Glass Beads and Water

As the shaking of mycelial mats growing on agar slopes led to the detachments of clumps of agar into the spore suspension, an experiment was carried out to measure the effectiveness of shaking a detached mycelial mat with glass beads and water.

Ten slopes of sporulating mycelia were taken and the sporulating mycelia removed and placed in loz universal bottles. A whirly mixer was used to spin the mat with 10ml of distilled water and a 1.5 ml depth of Ballotini beads for 10 seconds. The concentration of the spores in the water was then counted and is presented in

table 3.2. This procedure was also carried out with a 2 day old slope.

Table 3.2

THE CONCENTRATION OF SPORES ACHIEVED BY SHAKING DETACHED MATS OF 10 DAY OLD CULTURES OF C.BAINIERI WITH GLASS BEADS AND WATER

	Spore concentration (spores/ml)	
Replicate		
1	2.7×10^6	Mean = 3.4×10^6
2	2.9×10^6	Standard deviation =
3	4.1×10^6	0.88×10^6
4	3.6×10^6	
5	2.3×10^6	
6	2.5×10^6	
7	4.6×10^6	
8	4.0×10^6	
9	2.9×10^6	
10	4.7×10^6	

An identical method was used to extract spores from a 2 day old slope which produced a mean concentration of 1.2×10^5 spores ml^{-1}.

Discussion

This experiment showed that the number of spores which could be released from 2 day old cultures was approximately ten times lower that the number released from 10 day cultures. It also emphasised the variability

of spore production and release in cultures which were supposedly grown under identical conditions. A two fold difference in concentration between the highest and lowest spore concentrations was measured.

This method of spore release did prove to be more efficient than the previously tried methods by doubling the average total number of spores released. It also eliminated problems with agar which were noted in section 3.2.1.1, the only problem with suspended detritus being caused by mycelial fragments.

Microscopic examination of the material remaining after the spore removal procedure showed that the process was far from efficient, large numbers of spores remained in the mycelial mat. Liquid added to dry mycelial mats made the vertical hyphae collapse and wetting the hyphae and removal of the spores under the flattened layer was not easy. Washing and re extracting from mycelial mats was now examined.

3.2.1.3 Repeated Spore Extraction from the same Mycelial Mat

Microscopic examination of the mycelial mat after extraction of spores using glass beads indicated that many more spores were left attached to the mycelia than were released after one extraction. Quantification of the numbers of spores remaining on the mycelial mat was impossible. The huge numbers of spores remaining after any of the extraction procedures, were enmeshed in the mycelia at many different depths. Attempts were made to

assess the usefulness of multiple rinsings to increase spore harvesting efficiency.

Sporulating mycelia grown on malt extract broth in a petri dish were added to a 250ml Erlenmeyer flask with enough 1.5 to 2mm diameter Ballotini beads to cover the bottom of the shake flask. After adding 50ml of distilled water the flask was shaken at 250 rpm in a shaking incubator for 150 sec. The liquid was decanted from the shake flask, a further 50ml of water was added and the shaking repeated.

Results

An examination of the spore concentration in both the spore solutions showed that 5.2×10^6 and 4.9×10^6 spores ml^{-1} were produced respectively.

Discussion

The findings of the previous two experiments were confirmed by this experiment. Spores were released by shaking with water and glass beads, there were many spores left behind in the mycelial mat. Removal of the mat from an agar surface was very difficult because the mat was strongly anchored. Older cultures of C.bainieri (older than 2 days) seemed to anchor quite firmly in the top millimetre or so of the agar. Combined with the toughness of the mycelia this made dealing with mycelial mats grown on agar very undesirable.

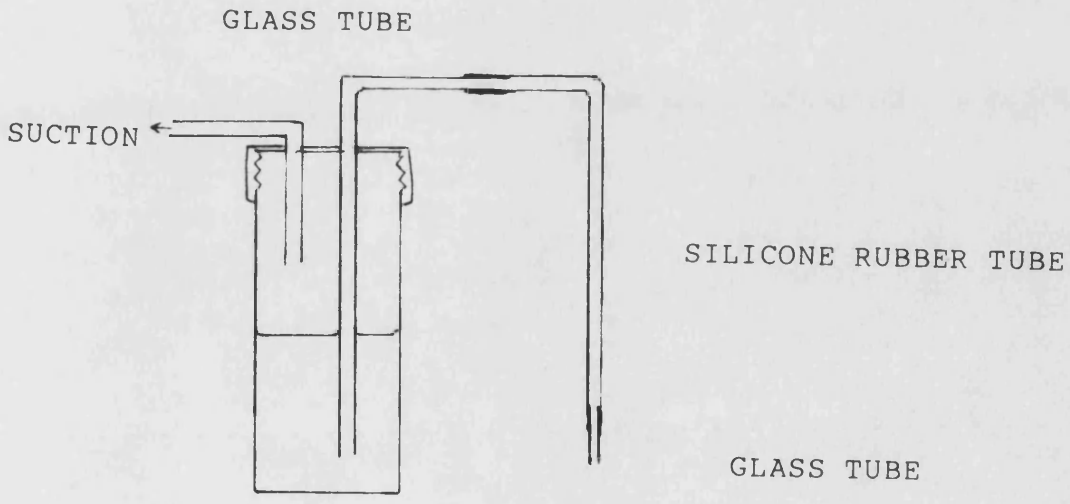
3.2.1.4 The Use of a Dry Suction Technique to Remove Spores from Mycelial Mats

An experiment was conducted to determine whether spores could be removed by using a dry suction technique to overcome the collapse of the aerial mycelia. This collapse took place whenever water or a solid object touched the mycelia.

The apparatus shown in figure 3.1 was used to try and collect spores from dry mycelial mats using suction. The collecting end was held at various distances between a centimeter and agar level above the sporing mycelia and slowly moved in ten replicate lines.

Figure 3.1

A Diagram of Equipment for the Collection of Spores by Suction



Results

No spores were removed from the mat unless the collecting tube was touching the mycelial surface. A collapse of upright aerial mycelia resulted in most of the spores being trapped underneath the mycelia. This, combined with the deposition of spores in the collecting tube this ensured that insufficient spores reached the collecting water to enable counting to be carried out.

Discussion

Experiments into the dry suction technique showed that the development of such a technique would require the investment of considerable time and effort before techniques using Ballotini beads and water could possibly be equalled work in this direction was terminated.

3.2.2 Spore Production

Available media were tested for their suitability as substrates for the heavy growth and sporulation of mycelial mats. The ease of removal of the mats was also of great interest, the aim was to produce large numbers of spores suitable for extraction by shaking with beads.

Four petri dishes containing each medium were assessed for growth and sporulation at 27^{o}C. The media used were tryptone soy broth, malt extract broth and malt extract broth with half and double the concentration of agar normally used to produce malt extract agar plates.

Results

Growth on half strength and double strength malt agar did not aid the removal of mycelia.

Good mycelial growth could be achieved by growing the fungus on a static liquid layer in petri dishes. Malt extract broth proved to be an ideal medium for this method. Tryptone soy broth produced poor mycelial growth.

Discussion

As expected the tryptone soy broth did not grow C.bainieri well. All the malt extract based media grew the fungus well. It was hoped that the fungus would not penetrate the surface of the double strength agar to the same extent as it penetrated the ordinary malt extract agar used. Unfortunately this did not appear to be the case as the mycelia were as difficult to remove from the double strength agar as from the single strength agar. The aim of using half strength agar - to reduce the cohesiveness of the growth medium (to make it sloppy) in order to facilitate the removal of the mycelial mat was also not attained. A decision was therefore made to use malt extract broth for the next stage of the work.

3.2.3 Spore Harvesting

Methods of producing and harvesting large concentrations of spores were investigated, with the objective of using these spores to inoculate several

Large Scale Spore Extraction 1

1 Take liquid grown mycelial mats from 7 large petri dishes.

2 Add to a sterile 500 ml bottle containing a 1 cm depth of glass beads (1.5 to 2.0 mm in diameter).

3 Add 100 ml of sterile distilled water.

4 Shake in a 500 cm arc 20 times.

5 Pour into 100 ml sterile centrifuge tubes.

6 Repeat stages 2 to 5 three times.

7 Centrifuge at 4^{o} C and 4000 rpm for five minutes.

8 Pour off supernatant.

9 resuspend spores in sterile distilled water.

10 Pool spores

11 Make up volume to 70 ml with sterile distilled water.

12 Repeat 7 to 11 twice.

13 After the final spin resuspend the spores in 17 ml of sterile distilled water.

Large Scale Spore Extraction 2

1 Take liquid grown mycelial mats from 4 large petri dishes.

2 Cut the mats into pieces using a sterile scalple and forceps.

3 Add the fragmented mycelial mats to two sterile 500 ml shake flasks containing a 1 cm depth of glass beads (1.5 to 2.0 mm in diameter).

4 Add 100 ml of sterile distilled water.

5 Leave 10 minutes.

6 Shake in an incubator at 250 rpm for 5 minutes.

7 Pour into 100 ml sterile centrifuge tubes.

8 Repeat stages 4 to 7 twice more.

9 Centrifuge at 4^{o} C and 4000 rpm for five minutes.

10 Pour off supernatant.

11 resuspend spores in sterile distilled water.

12 Pool spores

13 Make up volume to 70 ml with sterile distilled water.

14 Repeat 9 to 13 twice.

15 After the final spin resuspend the spores in 10 ml of sterile distilled water.

fermenter runs.

Figure 3.2 shows the methods used for large scale spore extraction and concentration.

Results

Procedure one and two produced 17ml of a $5.4x10^7$ spores ml^{-1} suspension and 10ml of a $3.6x10^7$ spores ml^{-1} suspension respectively. Both of the procedures took approximately 8 hours to carry out.

Discussion

Large concentrations of spores were obtained using the methods developed. Some of these spores were used as inocula but it was found that after several weeks that the spores lost viability. Microscopic examination showed that many had begun to germinate at $+4^oC$ in distilled water.

3.2.4 Spore Germination

Different methods of measuring spore viability were investigated so that it should be possible to add known numbers of viable spores to fermenters each time they were run.

A spore suspension from a 10 day old culture was tested in three different ways;

i) Spores were incubated on a counting slide in Robinsons medium for four hours and 45% of the spores were seen to produce germ tubes in that time.

ii) A very heavy inoculum (1ml of 1 x10^7 spores ml^{-1}) was added to 50ml of Robinsons medium in a shake flask and sampled after 4 hours incubation in standard conditions and 30% of the spores had produced germ tubes. As most of the germinating spores were seen to be forming clumps this figure is suspect because of the distribution of spores within the medium. Several larger clumps of spores containing at least 50 germinating spores were identified at other points on the counting slide. Because of the size and distribution of the spore clumps it was impossible to estimate error.

iii) Spore suspensions were placed on malt extract agar in a petri dish and incubated for 4 hours. Germination of 10 day old spores was in the region of 95 to 98%. Old spores from stored inoculum (3 months old) gave only 2% germination.

Discussion

Estimating the viability of microbial spores or cells is always difficult. As the results of this experiment show, viability may be a function of the medium used for culture. Complex media such as malt extract will often give good results (91), whereas less complete media such as the defined Robinsons medium may give lower germination and growth (91). The germination of spores on malt extract agar was settled upon as a measure of viability as it enabled spores to be viewed as individuals. Clumping created too much interference for

liquid growth techniques to be used.

Early signs of pellet formation such as the agglomeration of spores were of great interest. Spore counting prior to the four hour germination period revealed very few spore clumps. After four hours, germinated spores were observed in clumps. The germ tubes were only one or two spore diameters in length and not all the germ tubes were touching. Therefore it seems unlikely that this initial clumping was caused by the tangling of germ tubes. Ungerminated spores were not clumped and this also leads to the conclusion that the initial stages of pellet formation at least, were due to changes in the surface of the spores.

3.2.5 Conclusions

From these results it can be concluded that the best way to collect high numbers of spores of C.bainieri was to grow the sporulating mycelia on liquid medium, add them to a vessel containing a liquid and glass beads, shake vigorously, decant the liquid and repeat the shaking with a new volume of liquid several times.

3.3 Shake Flask Experiments

3.3.1 Introduction

A number of preliminary shake flask experiments were carried out to provide valuable basic information before larger scale culture was attempted. Very little work has been carried out on the fermentation of Cunninghamella spp on any scale. Most of the literature was concerned with the abilities of Cunninghamella spp to carry out reactions using xenobiotic compounds as a substrate (39,40,53,114,115). Because of this paucity of information the shake flask experiments were invaluable in aiding fermenter design.

3.3.2 Determination of the Relationship Between Glucose Concentration and Fungal Biomass

To gain useful information about the growth of microbial cultures it is necessary to follow the course of growth of the microorganism, either directly or indirectly (13,20,75,96,119). Methods that are used to follow cell growth include cell counting (42), viable cell counts (42,102), dry cell weight estimations (20), light absorbance methods (75) and nutrient uptake measurements (75). All of these methods have disadvantages and most of them are more suitable for the measurement of the growth of cultures of single celled organisms.

CHAPTER 3

Cell counting can only be used with organisms which reproduce by means such as binary fission or budding and produce discrete individual cells rather than chains or clumps. There is no indication of the state of the cells counted, the majority may be dead or dying, especially at the end of a batch culture. This system of assessing the growth of cultures is obviously of little use with filamentous organisms.

Viable cell counts try to get over the difficulties inherent in cell counting by using some estimation of the physiological state of the cells. When animal cells are counted it is common practice to dilute the cells with trypan blue solution, healthy cells will not stain but damaged cells will stain blue (47). Dilutions of microbial cultures may be spread on agar plates and the number of colonies formed counted, this has the disadvantage of being selective (102) and far from instant.

Dry cell weight measurements are used because the biomass of any type of organism can be assessed by this method. However, this method is non discriminating, dead and dying cells, medium constituents and the weight of any support materials will all be weighed. Different dry weights will be recorded for the same sample dried at different temperatures because of the evolution of carbon dioxide from some carbonates, differential loss of water and the loss of other volatile compounds such as volatile oils (20). Significant proportions of cellular material

may also be lost due to washing with liquids of the wrong osmotic potential (20). Dry cell weight estimations are time consuming if several samples are to be tested, the samples have to be large enough to allow accurate weighing and the drying process can take many hours (typically 24 to 48 hours in the case of C.bainieri samples). The variation between dry weights can be high.

Light absorbance techniques are useful for rapid estimation of cell numbers and dry weight. Trinci has shown that the dry weight of fungal cultures can be directly estimated using absorbance readings if the cultures are growing in the fluffy filamentous mode (124,125). Like most of the assay systems discussed these techniques will not give an estimation of the number of viable cells present and are not easily adapted for use with pelleted organisms.

One widely used technique is the indirect determination of biomass by measuring the uptake of some nutrient, commonly glucose. Once the initial calibration has been carried out to relate the nutrient uptake to some other feature such as increase in dry weight or cell number a rapid method of following the progress of a fermentation is available. This method is obviously subject to the errors inherent in the calibration procedure and the errors inherent in the periodic sampling of fermentation liquors. Conversely, following the uptake of nutrients is a better way of following the physiological activity of a culture, rather than

measuring cell numbers which may be largely dead or senescent.

On the basis of the above facts the correlation of glucose uptake with biomass expressed as dry cell weight was chosen as the most convenient and rapid method of monitoring fungal growth. Pelleted growth of C.bainieri allowed the measurement of dry weights of small volumes of culture only by the sacrifice of whole cultures. The removal of representative samples of biomass from the culture vessels (especially shake flasks) was impossible because of the distribution of the pellets in the medium (section 3.3.5). In larger vessels where the rapid removal of 100ml samples using wide bore tubing would present no significant effect on fermenter performance, direct dry weight estimations could be a reasonable proposal.

The correlation between glucose uptake and the dry cell weight was carried out twice, using two different methods of inoculation. Firstly the Sewell method was used (chapt 2) and secondly a spore inoculum was used (chapt 2).

3.3.2.1 Experiment DW1 A Comparison of Dry Cell Weight and Glucose Uptake Using Slope Inoculum

Twenty four flasks of secondary growth medium were inoculated with primary culture and grown under standard conditions as described in chapter 2. Four flasks were sacrificed at time zero and at 24 hour

periods after the initial sample time. Glucose and dry weight estimations were made over a five day period but unfortunately the glucose assay failed (fig 3.3 and table 3.3).

3.3.2.2 Experiment DW2 A Comparison of Dry Cell Weight and Glucose Uptake Using Spore Inoculum

Spore suspensions were used to inoculate 20 shake flasks as described in chapter 2. The shake flasks were sacrificed as in the above experiment.

3.3.3 The Effects of Antifoams On Growth and Codeine Conversion by C.bainieri

Sewell reported that the use of agitation speeds of greater than 700 rpm in the STR led to foaming when growing Cunninghamella spp (108).It was hoped to compare the growth of C.bainieri at different agitator speeds and an antifoam was necessary to enable the use of higher agitation speeds. A suitable antifoam had to be found and tested for its effects on growth and codeine conversion of the fungus.

A silicon antifoam was chosen for the purpose, as it was claimed that only low concentrations of this antifoam were required to control foaming (110). Many of the antifoaming agents considered were carbon based compounds such as vegetable oils which may have provided another carbon source for microbial growth (14). The antifoam chosen was tested as non ionic and anionic

emulsions.

Two experiments were carried out. Experiment one was designed to determine if there were any gross effects of antifoams on fungal growth or conversion of codeine. Experiment two was designed to assess whether there was any effect on growth, or conversion, by the most favourable antifoam compound during the expected duration of the fermentation. It was also designed to overcome problems caused by differing rates of growth observed during the first experiment. This was attempted by not sacrificing samples but by repeatedly sampling the same cultures over a period of time.

The methods on page 57 were used to inoculate and grow the cultures. Glucose, dry weight and norcodeine were estimated using the methods described in Chapter 2.

3.3.3.1 Experiment AF1 A Comparison of the Effects of Antifoams B and C on Growth and Codeine Conversion by C.bainieri

C.bainieri was grown in secondary cultures containing concentrations of 0,10,50 and 100ppm of antifoam B and a second set of shake flask cultures was grown containing the same concentration of antifoam C. Replicates of 3 shake flasks per concentration were used and the replicates were sacrificed after seven days. Dry cell weight, norcodeine concentration, glucose concentration and medium volume were measured. Visual observations of the type of growth during the

fermentation were also made.

3.3.3.2 Experiment AF2 A Comparison of Long Term Growth of C.bainieri in the Presence and Absence of Antifoam B

Five replicates of C.bainieri containing no antifoam were compared with five cultures containing 100ppm of antifoam B over 12 days of secondary culture. Two ml samples of culture medium were taken from the shake flasks after 0,4,6,8 and 13 days of culture.

3.3.4 Inoculum Experiments

3.3.4.1 Introduction

Experience with producing inocula for shake flask and STR experiments had shown that the method of inoculum production used by Sewell (108) was far from ideal for use with large fermenters or large numbers of shake flasks. The main objections to the Sewell method were that in the production of primary cultures using pieces of 2 day old slopes of C.bainieri the resulting cultures were very variable. Some cultures exhibited a rapid uptake of glucose whilst others grew very slowly and the morphology of the fungus in the cultures varied between pellets and filaments. Microscopic examination of medium from these cultures showed that the growth of pellets and filaments was due to the germination and growth of spores. It was also observed that good growth was more likely in a culture containing several cm^3 of malt agar from the slope. Once grown the primary cultures then had

to be transferred into flasks of secondary growth medium. In order to guarantee rapid secondary growth a dense primary culture must also be produced. Unreliable growth rate and growth form in primary cultures meant that a large amount of redundancy had to be built into the system to ensure that sufficient secondary cultures could be inoculated.

The use of secondary cultures to inoculate the STR was also very cumbersome as 400ml of primary culture was supposed to be added to the fermenter without special transfer equipment.

Three sets of shake flask experiments were carried out to try and determine the usefulness of spore suspensions as a source of inocula for primary cultures and fermenters. In all three experiments greater emphasis was placed on the history of individual cultures than the mean readings taken for each sample time.

3.3.4.2 IEl An Experiment to Compare Spore and Slope Inocula

This experiment was designed to determine whether spore suspensions could be used to sucessfully inoculate shake flasks with C.bainieri. It was also hoped to determine whether the growth kinetics of the spore and slope inoculated cultures were compatible.

Culture conditions, glucose assay techniques and preperation methods are all described in chapter 2. Two sets of replicate flasks were set up, four flasks were

inoculated using spore inocula (replicates 1 to 4) and the other four were inoculated using mycelia from a two day slope (replicates 5 to 8). One millilitre of spore suspension was added to each flask.

The spore suspension was produced by jetting 10ml of sterile distilled water containing 0.4% w/v tween 80 onto the surface of a four day old slope in a 1 oz glass bottle. The bottle was hand shaken through a distance of 30cm ten times and then whirlimixed for 10 seconds. As the surface of the mycelial mat seemed to be relatively undisturbed a pipette tip was used to scrape the surface. This treatment resulted in the release of 2.52×10^6 cells per ml.

Sampling of the cultures was carried out at approximately four hour intervals for the first 24 hours of the culture. Two milliliter samples of the culture medium were removed at these intervals using sterile 5 ml pipette tips which had been cut at the end to prevent clogging. Debris were removed by spinning 1.5 ml of the sample in a microfuge at the highest setting for two minutes.

3.3.4.3 IE2 A Comparison of the Effects of Spore Age and Concentration and Tween 80 on Inocula For Shake Flasks

The aims of this experiment were to identify the effects of several factors on inoculation using spores.

These factors were:-

1 spore concentration.

2 spore age.

3 the effect of tween 80 addition.

It was also hoped to achieve more consistent growth than was achieved in experiment IE1 and to compare pooled growth data with individual curves and growth kinetics with growth form. The course of growth in five replicate groups of four shake flasks were followed over a period of 36 hours using the same sampling technique used in the previous experiment. Spore production, release and inoculation of the cultures were carried out as follows.

i Spore Production

Large numbers of spores were produced by growing C.bainieri for 6 days at $27^{o}C$ on a malt agar slope in a 500 ml medical flat.

ii Spore Release

Spores were released by shaking the slope in the bottle with about 20g of glass beads of 2 to 3mm diameter and 30 ml of distilled water. This was not ideal as some agar soon slipped (30 sec) and became mashed. This produced a concentration of approximately 3×10^{6} spores ml^{-1} of liquid.

iii Inoculum Technique

Twenty shake flasks were inoculated in groups of four, the five groups being dealt with in the following ways:-

a) Shake flasks 1 to 4 had 1 ml of spore suspension added to each flask to produce a final concentration of 6.0 $x10^4$ spores ml^{-1}.

b) Shake flasks 5 to 8 had 2 ml of spore suspension added per flask. This gave a final spore concentration of 1.2 $x10^5$ spores ml^{-1}.

c) Shake flasks 9 to 12 had 0.1 ml of spore suspension added per flask to give a final spore concentration of 6.0 $x10^3$ spores ml^{-1}.

d) Shake flasks 13 to 16 were treated as flasks 9 to 12 but from the spores were suspended in a 0.1% tween 80 solution. This gave a final tween 80 concentration of 0.002% w/v.

e) Shake flasks 17 to 20 were inoculated using pieces of 2 day old slope (see chapt 2).

3.3.4.4 IE3 A Comparison of Washed Spores and Mycelial Mat as Inocula For Shake Flasks

This experiment was carried out using twenty four shake flasks (six replicates of four). The aims of experiment three were:-

1 To directly compare the effect of tween 80 and antifoam B on washed spores.

2 To compare the growth of cultures grown from washed

spores with the growth of cultures inoculated using agar and mycelia from petri dishes.

All the details of the experiment were as already described apart from the following procedures.

i Spore Inoculum

A quarter of the agar and mycelial mat from a ten day old culture of C.bainieri grown in a petri dish was placed into a 250ml Erlenmeyer flask with a 1 cm depth of 1 to 2 mm diameter glass beads. Fifty millilitres of sterile distilled water was added and the flask shaken for 2 minutes at 250 rpm. The extract from the flask was placed into two 50 ml sterile centrifuge tubes and the spores were spun down for 5 minutes at 400 rpm and $4^{o}C$ an an MSE Centaur 2 centrifuge. The supernatant was discarded and the spores were resuspended and spun down again. After discarding the supernatant the spores were resuspended and pooled.

ii Mycelial Mat

Sections of cultures from petri dish cultures were added directly to the culture medium. These were 1/8,1/4 and 1/2 of the total surface area of the culture including mycelia and agar.

iii Inoculum Technique

The replicates were inoculated as follows:-

a) Flasks 1 to 4 were inoculated using a quarter of a petri dish.

b) Flasks 5 to 8 were inoculated with a half petri dish each.

c) Eighth sections of petri dish cultures were used to inoculate flasks 9 to 12.

d) Flasks 13 to 24 were inoculated with 0.5 ml of spore suspension per flask. The initial concentration of the spore suspension was 3.7×10^6 and hence the spore concentration was 3.7×10^4 in the flasks. Only washed spores were used to inoculate flasks 13 to 16. Flasks 17 to 20 were inoculated and then antifoam B was added to a concentration of 100ppm. Flasks 21 to 24 were inoculated and tween 80 was added to a concentration of 0.1% w/v.

3.3.5 Experimentally Observed Growth Forms in Shake Flasks

3.3.5.1 Attached Growth

Rings of microorganism formed at the limits of nutrient height. These started forming very early in the fermentation and when spore inocula were used, a visible ring of germinating spores could be detected by eye after three or four hours. Ring growth could be prevented by stopping the shaker and swirling each flask individually to wash down stranded material. It was impractical to do

this every four hours or so with cultures which continued for periods in excess of ten days. In the longer term cultures rings were washed down once a day. This resulted in the formation of quite large pellets (1 to 2 cm in diameter). The presence of tween 80 (0.002% w/v) in the medium reduced ring formation, probably as a result of the lowered surface tension of the culture medium, thus achieving the same effect as siliconising the walls of the vessel (127).

A second form of attached growth occurred as a thin layer of fungus growing on the inner surface of the cotton wool plug of the shake flask. This layer must have formed as a result of splashing of spores and medium onto the cotton wool.

The major form of attached growth in the shake flasks was the ring. If left unchecked ring growth can become the dominant growth type in the shake flask (127). This is not just a result of straight forward growth the ring can continually accumulate new material from the medium. Periodic removal of the ring material by wiping in a shake flask culture was shown to result in total depopulation of the medium (127). It could not be determined if the ring took part in the formation of small pellets but it certainly aided the formation of larger pellets in these experiments.

3.3.5.2 Pelleted Growth

Pelleted growth took place in approximately three

out of four of the primary cultures and all the secondary cultures used. The size of pellets varied between and within the flasks. Glucose uptake data for individual shake flasks confirm this difference between flasks (3.3.6). In early work the difference between the number and size of pellets was extreme in some cases. One flask exhibited only a single large pellet whilst others from the same batch contained many pellets. The variations observed early in the project led to the investigation of the effect of inoculum on growth form described in section (3.3.4). When spore suspensions were used to inoculate shake flasks and then samples were taken the spore concentration in the samples was always far less than the calculated value.

Germinating spores when found were normally in clumps of 3 to 10 whilst the non germinating spores usually floated free.

As the results in section (3.3.6) show, pelleted growth resulted in uneven growth of C.bainieri. Formation of larger pellets could be a result of wash down from the flask wall, pellet growth or accumulation of pellets caused by some other factor. Germinating spores exhibited a great tendency to clump, it was not determined whether this was due to a change in the electrostatic charges on the pellets or the tangling of germ tubes. Evidence suggests that both types of pellet formation are have occurred.

Treatment of cultures with tween 80 resulted in

the formation of smaller than normal pellets and a lack of ring formation. This correlation could be due to:-

i A lessening of the attraction of germinating spores for each other.

ii lowering the residence time of spores at the air liquid vessel interface.

iii Alteration of the morphology and physiology of the fungus.

There was no time to investigate this further but the evidence points towards the first two reasons being important.

The combination of lower spore concentrations than expected in the medium, early ring formation and the absence of non germinating spores from very small pellets suggests that pellet formation occurs after germination. It was not possible to determine by microscopic examination whether the small pellets were formed in the medium or at the site of ring formation. However it is certain that small pellets accumulated in the ring area after a very short period of fermentation. Small pellets in the ring region were also observed to grow until several pellets formed a larger pellet. Patel (95) has found that siliconisation of the walls of shake flasks results in a much reduced ring formation and proliferation of small pellets in C.bainieri.

3.3.5.3 Mycelial Growth

Mycelial mats and spore concentrations of up to

5×10^6 spores ml^{-1} produced filamentous mycelial growth in approximately one out of four cases. The only sure method of producing this growth form was to use an inoculum of sporing mycelia on its malt agar growth medium. Glucose utilisation in filamentous cultures was much quicker than in pelleted cultures, possibly because the lag phase was shorter and because growth was logarithmic, not cubic. Microscopic examination of these cultures showed many spores with short germ tubes.

As the results in section (3.3.6) show, pelleted growth resulted in uneven growth of C.bainieri. Formation of larger pellets could be a result of wash down from the flask wall, pellet growth or accumulation of pellets caused by some other factor. Germinating spores exhibited a great tendency to clump, it was not determined whether this was due to a change in the electrostatic charges on the pellets or the tangling of germ tubes. Evidence suggests that both types of pellet formation occurred.

The factors determining growth type were not identified but some mechanical factor such as small agar particals may have aided in preventing the production of large pellets. Even at the end of mycelial fermentations the mycelia were quite short, no more than several spore lengths and this may mean that the only reason that pellets were not formed was that the growth medium was exhausted before small pellets could increase in size.

3.3.6 Results and Discussion

Experiments DW1 etc have been described in sections 3.3.2 to 3.3.4 inclusive and are discussed in the same order.

3.3.6.1 Glucose and Dry Cell Weight Relationships

Experiment DW1

The first set of dry cell weights found by using shake flasks inoculated from a primary culture were very variable. The mean data points could be fitted equally well to a straight line or a sigmoidal curve (fig 3.3). These results were useless as a means of correlating glucose uptake with dry cell weight and emphasise the potential for variability in fungal cultures.

Experiment DW2

Dry cell weight replicates had a smaller standard deviation than the replicates used for the first run (fig.3.4). At first sight this may not appear so but the scale on figure 3.4 is larger than the scale on figure 3.3. As can be seen from figure 3.5 the ratio of dry cell weight to glucose utilised is not constant over time. The largest anomaly occurred at the lower end of the time scale. After 24 hours of growth the ratio was less than 0.075. This can be explained if it is assumed that the energy requirements per unit mass of germinating spores are greater than those of fungal pellets. As can be seen from the introduction this is a reasonable

assumption to make as the whole of the surface of the germinating spores was available for nutrient uptake unlike larger particles such as pellets. Pellets only have a peripheral zone capable of nutrient uptake and rapid metabolism. Accumulation of slowly metabolising biomass in the inside of the pellets was probably the cause of the rapid rise of the ratio to around 0.5 and the slight drop in the value at the end of the run due to autolysis of the central portion of the pellets.

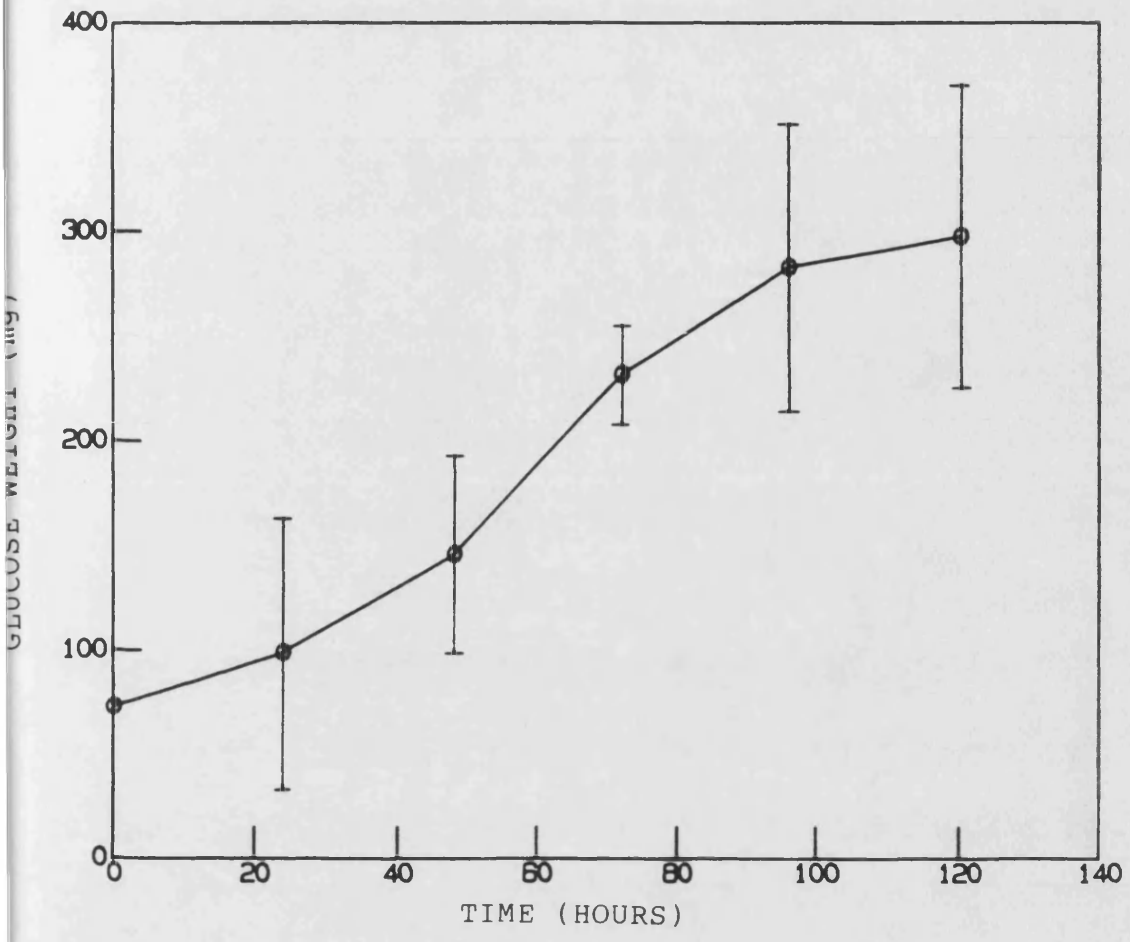
This experiment showed that after spore germination and initial growth had occurred the dry cell weight of fungi in a fermenter could be estimated using glucose uptake data. One gram of glucose produced approximately 0.5g of dry cell weight.

FIGURE 3.3

Dry weight (mg) against time for replicate shake flasks inoculated using slope inoculated primary culture (Expt DW1). Four flasks were sacrificed each day.

FIGURE 3.4

Dry weight (mg) against time for replicate shake flasks inoculated using spores (Expt DW). Four flasks were sacrificed each day.


400
300
200
100
0
0
20
40
60
80
100
120
140
TIME (HOURS)

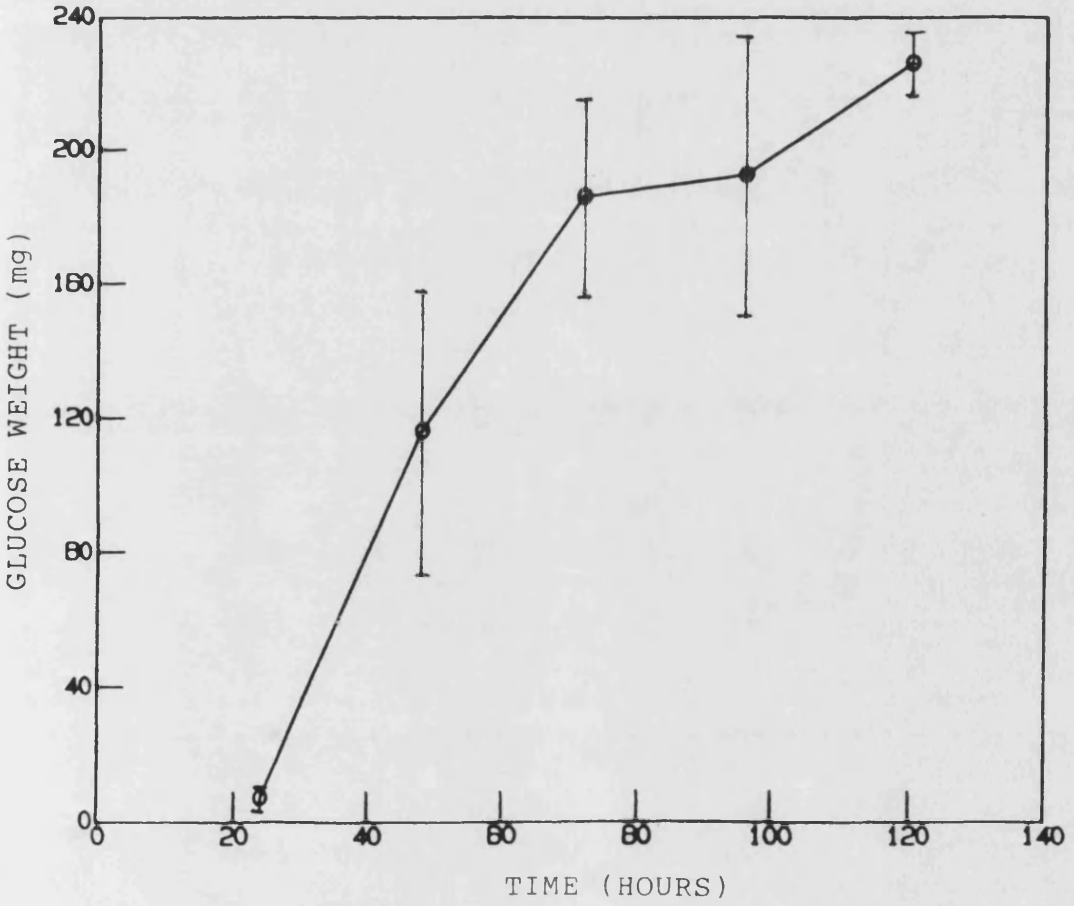
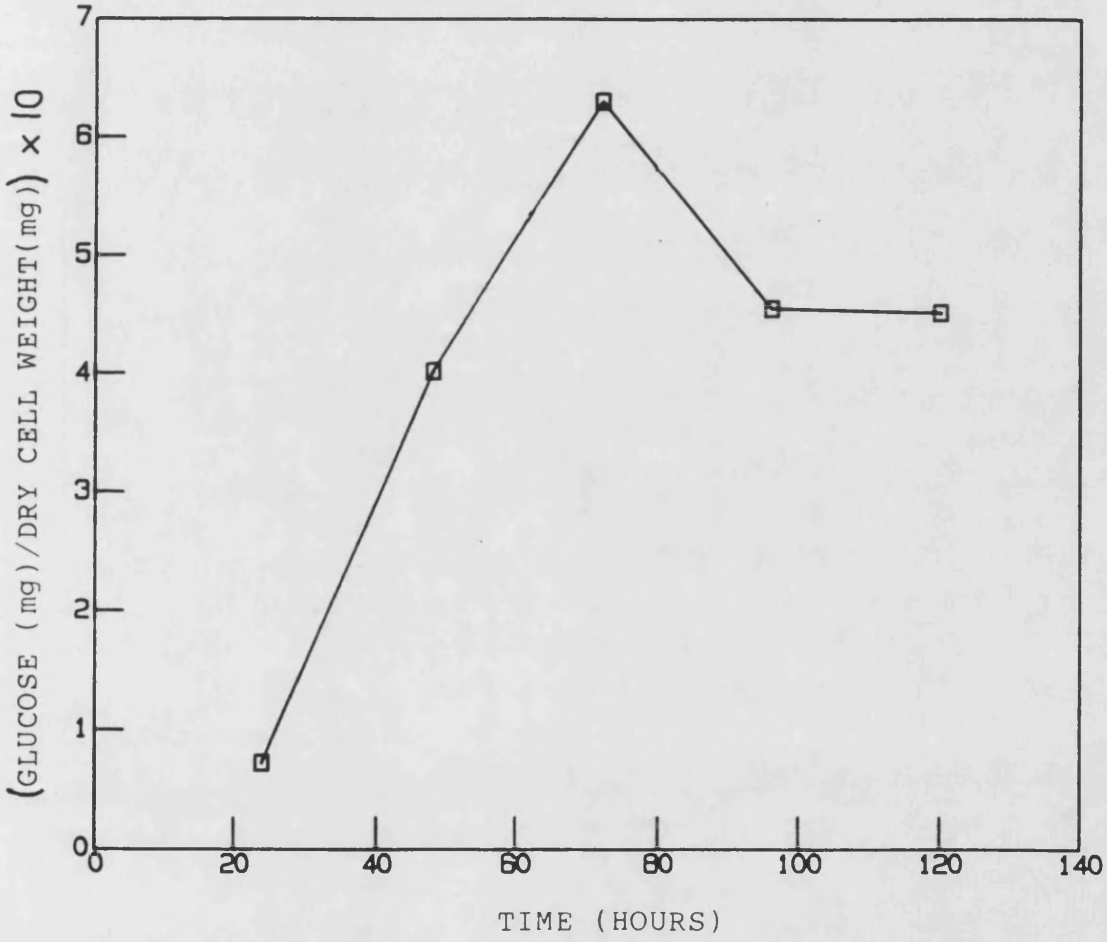

240
200
160
120
80
40
0
GLUCOSE WEIGHT (mg)
0
20
40
60
80
100
120
140
TIME (HOURS)

Figure 3.5

The Ratio of (Glucose Utilised to Dry Cell Weight) x10 Against Time for C.bainieri Cultures Inoculated with Spores and Grown in Shake Flasks



3.3.6.2 Antifoam Control Experiments

Experiment AF1

1) There was no obvious gross effect on the conversion of codeine to norcodeine by the addition of antifoams B and C (tables 3.3,3.4).

2) The method of producing primary cultures was inadequate. There was too much opportunity for variation.

Growth of primary cultures was very variable, some cultures producing cloudy filamentous growth but most producing a limited number of pellets with the size of pellets varying between different shake flasks. The antifoam B and C replicates were grown in identical conditions and the inoculum was the only variable between the two runs. In the antifoam B run all the glucose in the medium was utilised after 7 days (table 3.3). Only three replicates in the antifoam C run had utilised all their glucose after 7 days (table 3.4). This was despite using the same inoculum technique for both runs.

Once the secondary culture was inoculated no further pellet production could be seen to occur.

3) Variations between norcodeine produced were large i.e. a 7.4 fold difference was found between the lowest control value and the highest in the antifoam B control. This was probably because of differences in the time at which glucose was exhausted by different cultures or

because of the differences in fungal area available for the uptake and conversion of codeine. Both of the above factors are a function of the porosity and surface area of the pellets.

4) A dense inoculum of small filaments produced faster growing cultures which gave more reproducible results.

Experiment AF2

The second run was carried out using a well dispersed filamentous inoculum. As can be seen from tables 3.5 and 3.6 and there is no difference between the two treatments. It was noticeable that the dry weights recorded for this experiment were much more reproducible than in the first run.

TABLE 3.3

A TABLE TO COMPARE ANTIFOAM B CONCENTRATION WITH DRY WEIGHT, GLUCOSE UTILISATION AND NORCODEINE CONCENTRATION AFTER 7 DAYS SECONDARY FERMENTATION IN SHAKE FLASKS

Antifoam concentration (ppm)	Dry cell weight (mg)	Mean dry cell weight (mg)	Total norcodeine (µg)	Mean weight of norcodeine (µg)	Glucose (mg)
-	257	-	327	-	0.0
0	259	255	906	1219	0.0
-	251	-	2424	-	0.0
-	226	-	1544	-	0.0
10	236	231	1296	1484	0.0
-	229	-	1611	-	0.0
-	225	-	1480	-	0.0
50	227	232	824	875	0.0
-	245	-	321	-	0.0
-	225	-	1585	-	0.0
100	217	221	1482	1609	0.0
-	222	-	1759	-	0.0

TABLE 3.4

A COMPARISON OF ANTIFOAM C CONCENTRATION WITH DRY WEIGHT, GLUCOSE UTILISATION AND NORCODEINE CONCENTRATION AFTER 7 DAYS SECONDARY FERMENTATION IN SHAKE FLASKS (EXPT. AF1)

Antifoam concentration (ppm)	Dry cell weight (mg)	Mean dry cell weight (mg)	Total norcodeine (µg)	Mean weight of norcodeine (µg)	Glucose (mg)
-	225	-	200	-	0.000
0	223	224	234	145	2.889
-	224	-	000	-	6.644
-	119 *	-	1511	-	21.480
10	226	155	146	099	0.000
-	105	-	000	-	21.650
-	80	-	103	-	22.070
50	254	193	160	176	0.000
-	243	-	264	-	0.473
-	142	-	108	-	13.190
100	241	202	191	147	0.173
-	222	-	142	-	5.630

* most of the biomass in this flask was present as 1 large clump

TABLE 3.5

A Table of Results to Correlate Norcodeine Production and Time (Days) for Cunninghamella bainieri Grown in Modified ELJ Medium in the Presence and Absence of 100ppm of Antifoam B at 27°C and 250 RPM.

(Expt. AF2)

TIME(DAYS)	4	6	-	8	-	12	-
REPLICATE	NC	NC	-	NC	-	NC	-
1	ND	40.4	-	57.2	-	80.8	
2	ND	30.3	SD=10.4	48.8	SD=19.3	176.9	SD=52.4
3	ND	42.1	-	67.3	-	224.0	
4	ND	58.9	M=43.8	94.3	M=62.9	176.9	M=167.1
5	ND	47.1	-	47.1	-	176.9	
6	ND	20.2	-	65.7	-	132.6	
7	ND	40.4	SD=9.2	47.1	SD=7.7	167.7	SD=32.9
8	ND	33.7	-	50.5	-	211.9	
9	ND	38.7	M=35.4	48.8	M=52.2	135.6	M=165.5
10	ND	43.8	-	48.8	-	179.9	

KEY

M=Mean of the five replicate

SD= Standard deviation of the five replicates.

NC= The norcodeine concentration per flask ($\mu g\ ml^{-1}$)

TABLE 3.6

A Table of Results to Correlate Norcodeine Production Time (Days) for Cunninghamella bainieri Grown in Modified ELJ Medium in the Presence and Absence of 100ppm of Antifoam B at 27°C and 250 RPM. (Expt. AF2)

REPLICATE	G4	G6	DCW12	NCWT	NCWT/DCW
1	1.0	ND	192.3	646.3	3.36
2	2.8	ND	178.2	1415	7.94
3	0.1	ND	199.0	1792	9.01
4	0.2	ND	198.0	1415	7.15
5	0.2	ND	180.2	1415	7.85
6	1.4	ND	193.4	1061	5.49
7	2.7	ND	194.3	1341	6.96
8	3.6	ND	202.9	1695	8.35
9	3.6	ND	208.8	1085	5.20
10	3.0	ND	199.9	1493	7.20

KEY

G4= Glucose concentration after 4 days (mg ml^{-1}).

G6= Glucose concentration after 6 days (mg ml^{-1}).

DCW12= Dry Cell weight at end of fermentation (mg).

NCWT= Total weight of norcodeine produced after 12 days fermentation (µg).

NCWT/DCW= The weight of codeine per mg of dry cell weight (µg mg^{-1}).

The mean glucose concentration for replicates 1-5 after 4 days fermentation was 0.86 mg ml^{-1} with a standard deviation of 1.14.

The mean glucose concentration for replicates 6-10 after 4 days fermentation was 2.86 mg ml^{-1} with a standard deviation of 0.90.

The mean dry cell weights for replicates 1-5 after 12 days fermentation were 189.5 mg with a standard deviation of 9.8.

The mean dry cell weights for replicates 6-10 after 12 days fermentation were 165.5 mg with a standard deviation of 32.9.

3.3.6.3 Inoculum Experiments

Experiment IE1

Glucose Assay

Although the spores were seen to germinate after four hours in most cases glucose uptake was not detected until a further eight hours had passed (figs 3.6-3.11). Earlier experiments and not enabled the determination of the growth kinetics of the fungus. As can be seen from figures 3.6 - 3.11 the growth of the fungus was probably of logarithmic type after a period of about 12 hours and a cube root type after 20 hours. Most of the glucose in the medium was utilised after 72 hours.

One anomaly in the progression from logarithmic to cube root phase was culture 5. This exhibited two different cube root phases which changed at about 20 hours (fig 3.11).

In some cases it was not possible to determine whether logarithmic or cube root kinetics were occurring because of the timing of sampling. Several interesting points were noted:-

i The slope inoculum produced growth which best exhibited logarithmic and cube root kinetics figs.

ii Glucose utilisation by all but one of the spore inoculated cultures was almost completed within 47 hours of inoculation. Only one of the slope inoculated cultures had completed fermentation.

iii The growth form of culture 5 must have been dense

flocs to account for its cube root kinetics, the change in slope of the curve being caused by accumulation of the flocs and a reduction in the number of metabolising units in the medium. This process was probably aided by the process of mycelial deposition at the limits of the medium circulation height.

iv Analysis of individual flasks provided much better information about growth than the analysis of pooled data.

FIGURE 3.6

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension (Expt IE1). Flask 1 (○), flask 2 (●), flask 3 (⊟), flask 4 (⏚).

FIGURE 3.7

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a slope inoculum (Expt IE1). Flask 5 (○), flask 6 (●), flask 7 (⏚), flask 8 (⊟).

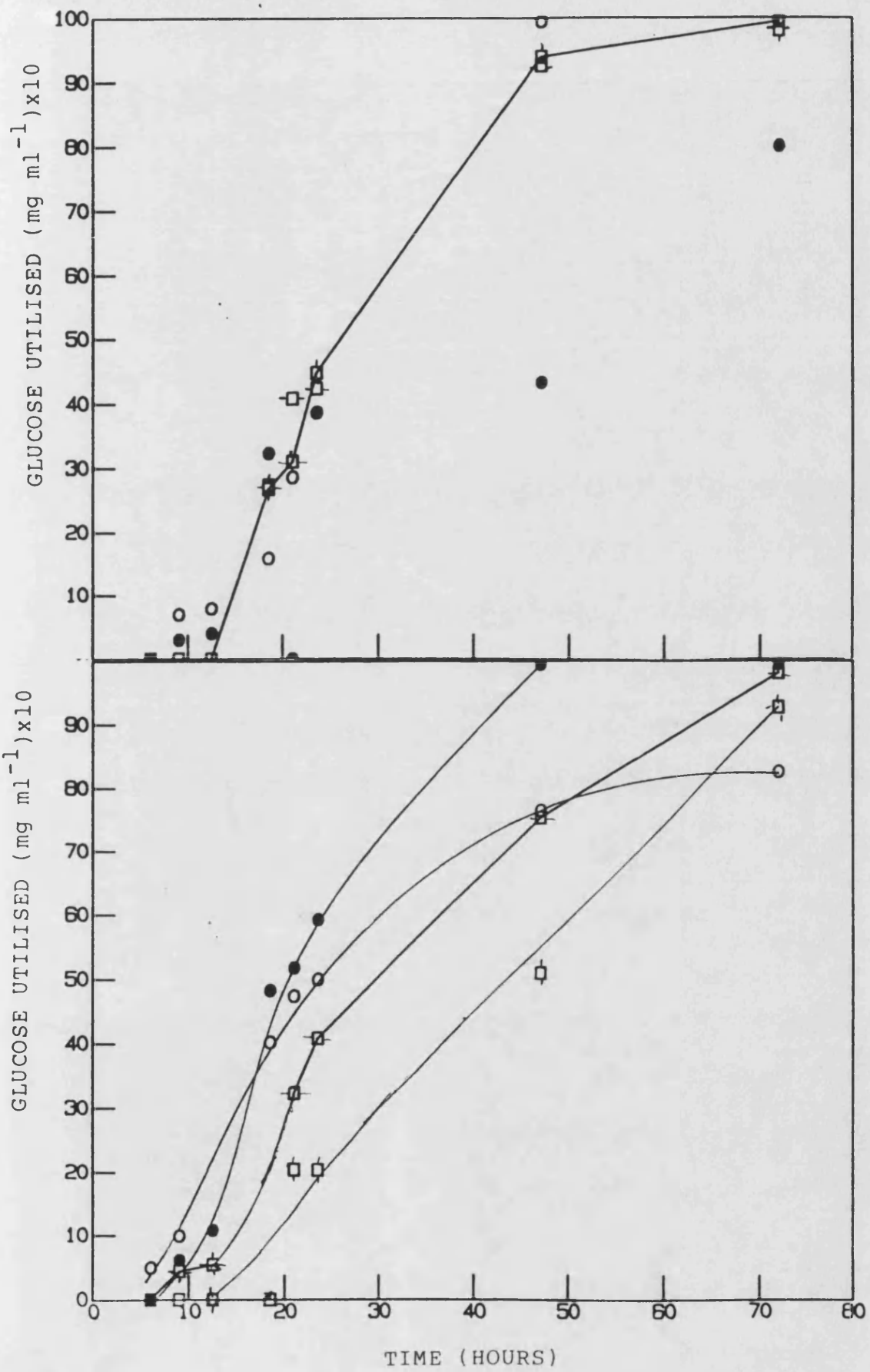

GLUCOSE UTILISED (mg ml^{-1})x10
100
90
80
70
60
50
40
30
20
10
GLUCOSE UTILISED (mg ml^{-1})x10
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
TIME (HOURS)

FIGURE 3.8

Log of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension (Expt IE1). Flask 1 (○), flask 2 (●), flask 3 (⊟), flask 4 (⏀).

FIGURE 3.9

Log of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a slope inoculum (Expt IE1). Flask 5 (○), flask 6 (●), flask 7 (⏀), flask 8 (⊟).

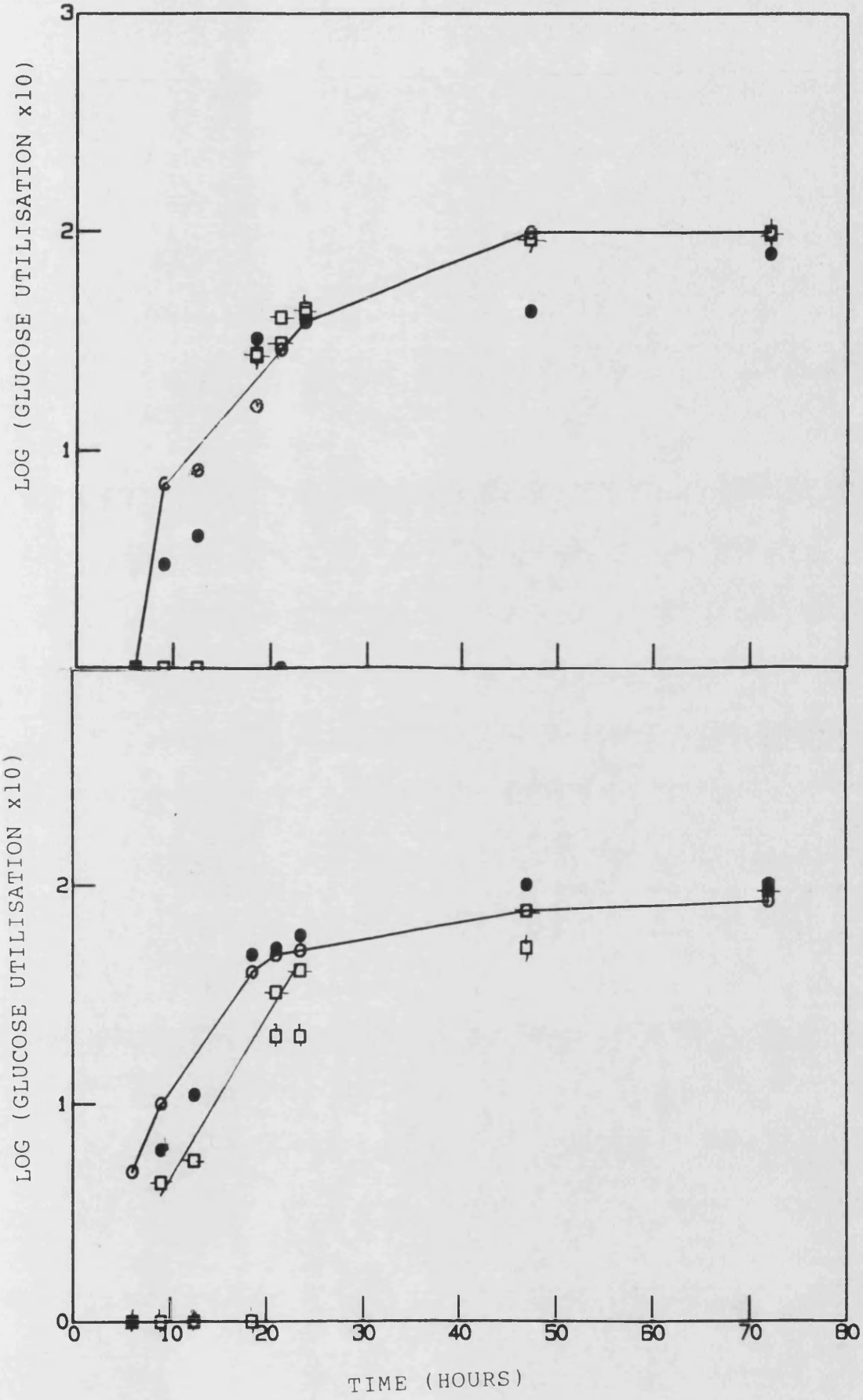
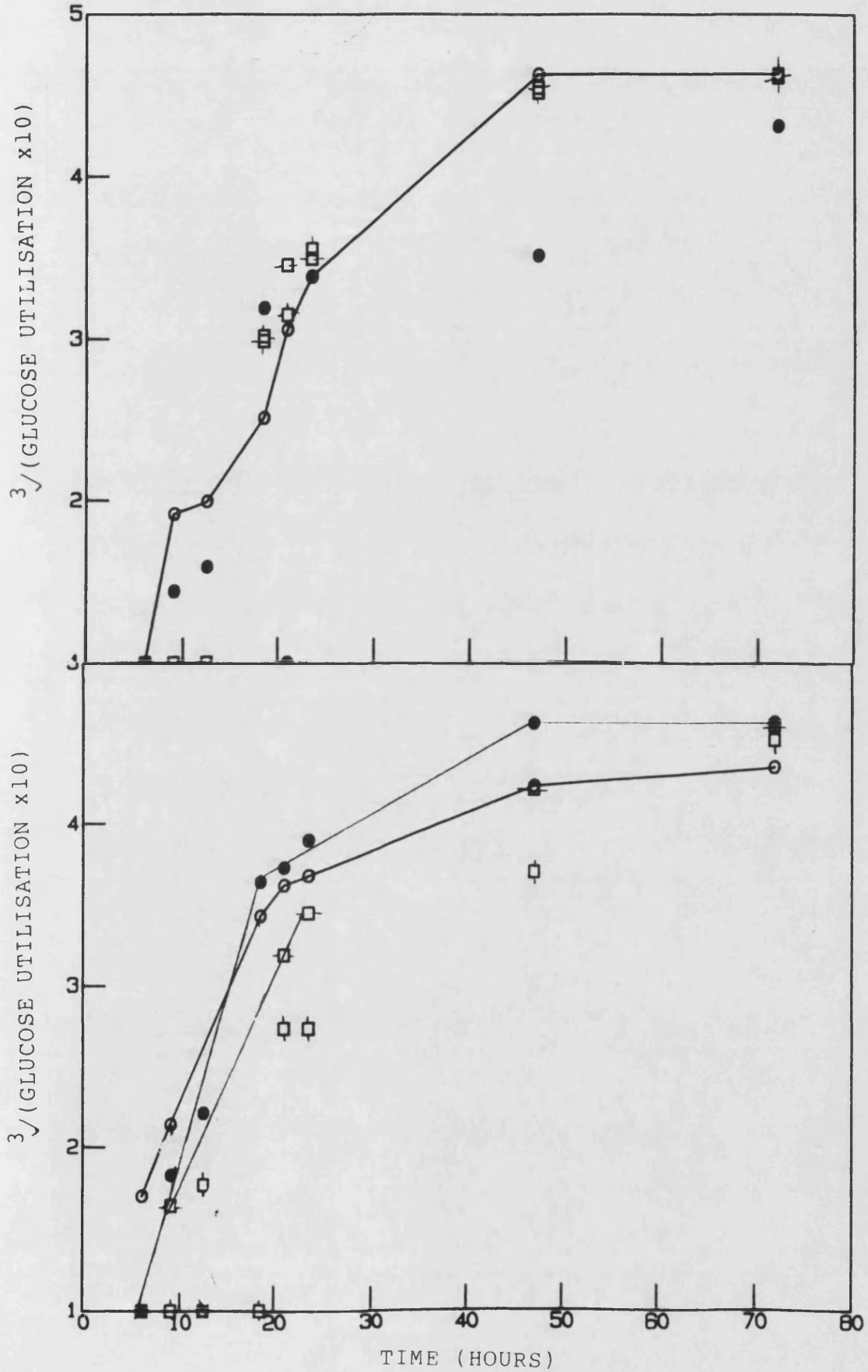

LOG (GLUCOSE UTILISATION x10)
3
2
1
LOG (GLUCOSE UTILISATION x10)
2
1
0
0
10
20
30
40
50
60
70
80
TIME (HOURS)

FIGURE 3.10

Cube root of glucose uptake (mg ml^{-1}x10) against time for four replicate shake flasks inoculated using a spore suspension (Expt IE1). Flask 1 (O), flask 2 (●), flask 3 (⊟), flask 4 (⏀).

FIGURE 3.11

Cube root of glucose uptake (mg ml^{-1}x10) against time for four replicate shake flasks inoculated using a slope inoculum (Expt IE1). Flask 5 (O), flask 6 (●), flask 7 (⏀), flask 8 (⊟).


$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
5
4
3
2
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
4
3
2
1
0
10
20
30
40
50
60
70
80
TIME (HOURS)

Experiment IE2

Results and Discussion

Several points can be made from the results shown in table 3.7 and figures 3.12 - 3.26. These points are:-

i Of five groups of replicates only one (replicates 13 to 16) produced similar growth curves in all four flasks. In the other groups the data was spread quite dramatically.

ii Cases of filamentous or small loose textured pelleted growth produced rapid decreases in glucose concentration. When analysed these growth curves tended to show a cube root relationship at the top of the curve and a logarithmic relationship lower down the curve. It was also noticeable that the cube root relationship did not hold when approximately 60% of the glucose had been utilised.

iii Larger pellets and small dense pellets utilised glucose slowly compared to the filamentous growth and small dense pellets. The cultures often exhibited a cube root relationship.

iv Although the growth in the shake flasks began with a uniform pulpy morphology the cultures soon began to differentiate.

v The formation of rings of mycelia above the medium occurred rapidly, starting within 3 or 4 hours of the fermentation. Only replicates 13 to 16 were free of rings.

vi Different spore concentrations did produce detectable differences in growth rates but the difference was only detected between the lowest spore concentration and the other spose concentrations. Replicates 9 to 12, the samples with the lowest spore concentration mostly showed very poor growth (figs 3.15-3.17). These cultures were also the only cultures not to exhibit any sign of mycelial growth after 24 hours of growth. They did, however, exhibit two types of growth curve with replicates 9 and 10 taking up little glucose in a manner similar to replicates 1 to 4.

Replicate 12 utilised glucose very quickly. It seems that low spore concentrations can produce rapidly growing cultures spontaneously.

vii Replicates containing 0.02% of tween 80 (13 to 16) showed the most reproducible growth, all four growth curves were very similar (figs 3.21-3.23). Small pellets and filaments were observed throughout all of the growth period and the mycelial rings present in the other flasks were not present.

Glucose uptake by the cultures containing tween 80 was uniformly high with 60% depletion after 36 hours of culture. This is in very marked contrast to the poor growth of samples 9 to 12 which were inoculated with an equal spore concentration. The

slope inocula showed good growth as well (figs 23-25)

viii The curves obtained by using 6 day old cultures as a source of spores were very similar to those obtained using 4 day old cultures as a source of spores.

ix The cultures containing tween 80 foamed more than the other cultures. There was a lag phase of approximately 12 hours before the detectable growth of almost all of the cultures.

Using the points above a description of how the growth of C.bainieri developed over the 36 hour period was formed.

An initial 3 to 4 hour lag phase as described for experiment one occurred, followed by the germination of spores to produce a cloudy culture. During the next eight hours the glucose uptake by the cultures was so small as to be undetectable but pellet formation was already occurring (table 3.7). The formation of pellets was related to the presence of mycelial rings at the limit of the shaken medium level. Detectable glucose uptake first occurred when the formation of denser pellets was still happening. The rate of glucose uptake was dependent on the culture morphology. If a low enough glucose concentration was reached by the time the fermentation was reached (40 mg ml^{-1}) the rate of glucose uptake slowed.

The above picture was totally in agreement with

the models of fungal growth described in chapter 1. A lack of logarithmic or cube root kinetics during the early and later stages of of the fermentations was easy to reconcile with the models. The accumulation of pellets causing a gradual reduction in number of the growing pellets during the early stages of fermentation, the production of irregularly shaped ring fragments and inhibition of growth by toxic metabolites or lowered glucose concentration could all affect the growth kinetics of the cultures.

TABLE 3.7
Growth Form of Cultures in Shake Flasks During IE2

FLASK	TIME (H) 12	16	20	24
1	LP	LP	LP	P
2	M,LP	M	M	M
3	LP	LP	LP	P
4	M,LP	M	M	M
5	LP	M	M	M
6	M,LP	LP,P	LP,P	P
7	M,LP	LP	LP	P
8	LP	LP	LP	P
9	LP	P+M	P,M	SMALL
10	LP	P,M	P,M	DENSE
11	SP,M	P,M	P,M	P
12	SP,M	P,M	P,M	
13	SP,M*	P,M*	P,M*	SP*
14	SP,M*	P,M*	P,M*	SP*
15	M,LP*	P,M*	P,M*	M*
16	LP*	P,M*	P,M*	M*
17	M,LP	P	P	P
18	M	M,P	M,P	M
19	M,LP	M,P	M,P	P
20	LP	M,P	M,P	P

KEY
M = Mycelial growth
P = Tight pellet
LP = Loose pellet
S = Small pellet
* Indicates that no mycelial ring was present in the shake flask.

FIGURE 3.12

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 6 $x10^4$ spores ml^{-1} (Expt IE2). Flask 1 (○), flask 2 (●), flask 3 (□), flask 4 (■).

FIGURE 3.13

Log of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 6 $x10^4$ spores ml^{-1} (Expt IE2). Flask 1 (○), flask 2 (●), flask 3 (□), flask 4 (■).

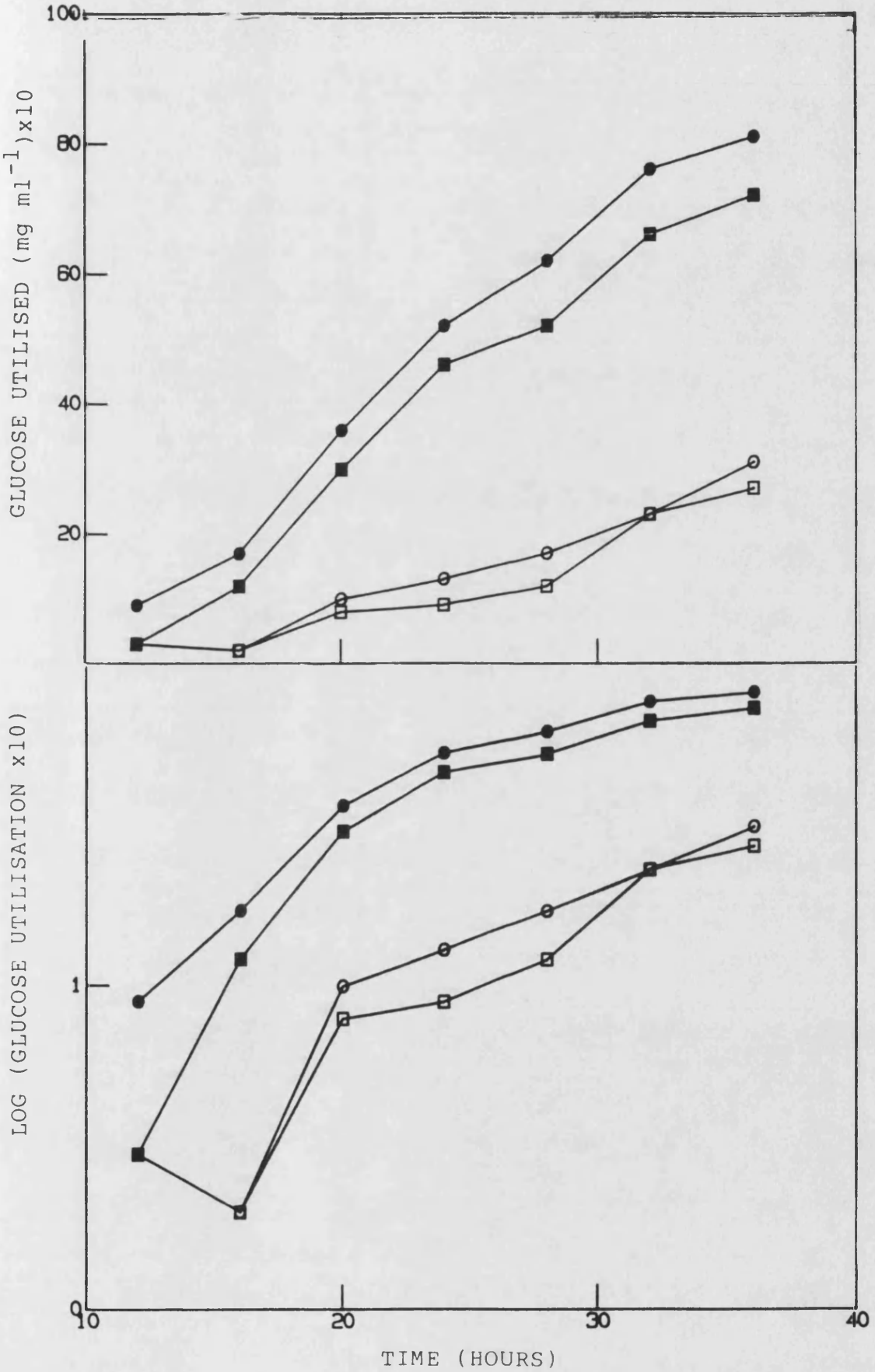

100
80
60
40
20
GLUCOSE UTILISED (mg ml^{-1})x10
LOG (GLUCOSE UTILISATION x10)
1
0
10
20
30
40
TIME (HOURS)

FIGURE 3.14

Cube root of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 6 $x10^4$ spores ml^{-1} (Expt IE2). Flask 1 (○), flask 2 (●), flask 3 (□), flask 4 (■).

FIGURE 3.15

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 1.2 $x10^5$ spores ml^{-1} (Expt IE2). Flask 5 (○), flask 6 (●), flask 7 (ϕ), flask 8 (⊡).

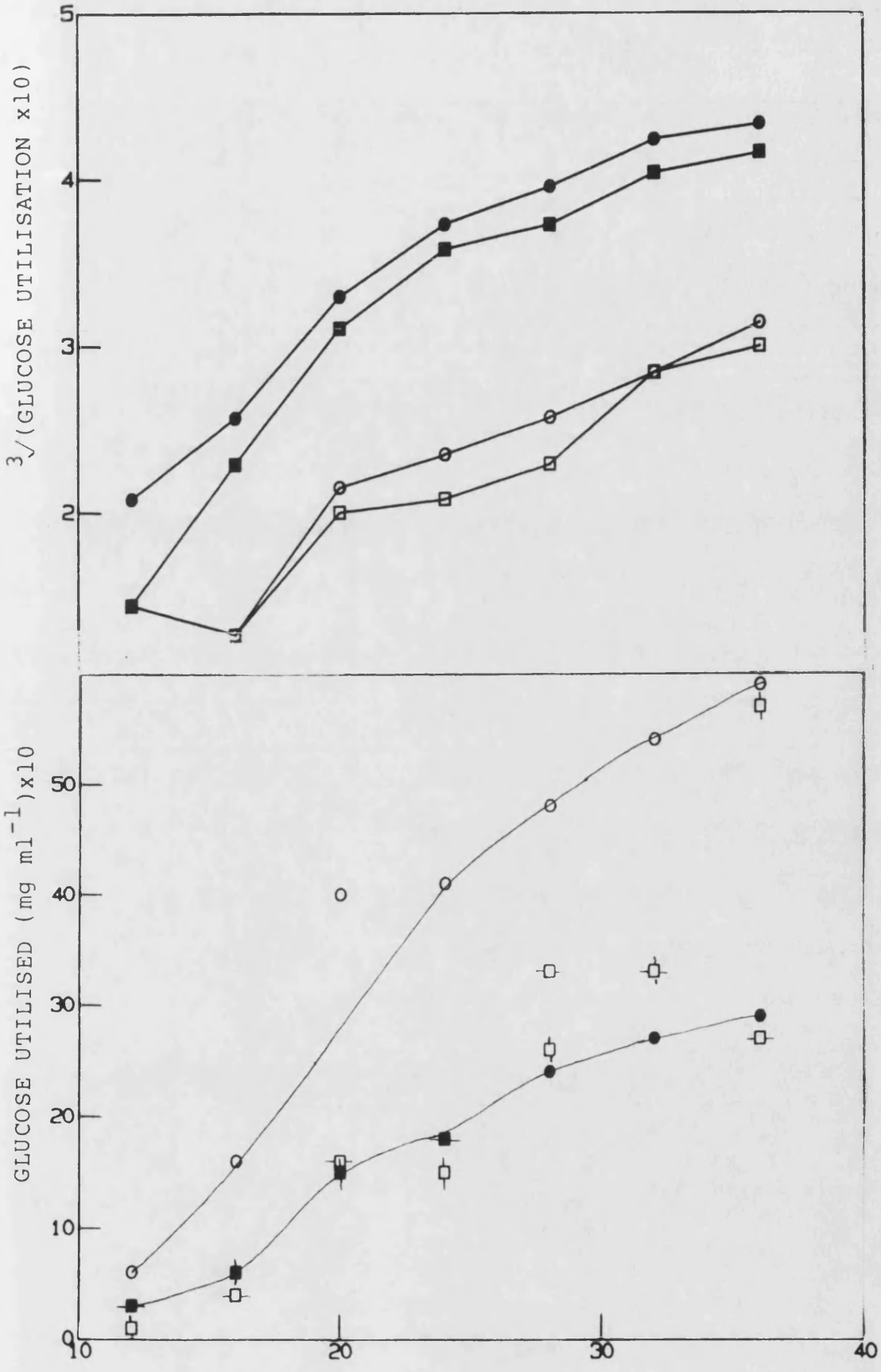

$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
5
4
3
2
GLUCOSE UTILISED (mg ml^{-1})x10
50
40
30
20
10
0
10
20
30
40
TIME (HOURS)

FIGURE 3.16

Log of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 1.2 $x10^5$ spores ml^{-1} (Expt IE2). Flask 5 (o), flask 6 (●), flask 7 (⏀), flask 8 (⊟).

FIGURE 3.17

Cube root of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 1.2 $x10^5$ spores ml^{-1} (Expt IE2). Flask 5 (o), flask 6 (●), flask 7 (⏀), flask 8 (⊟).

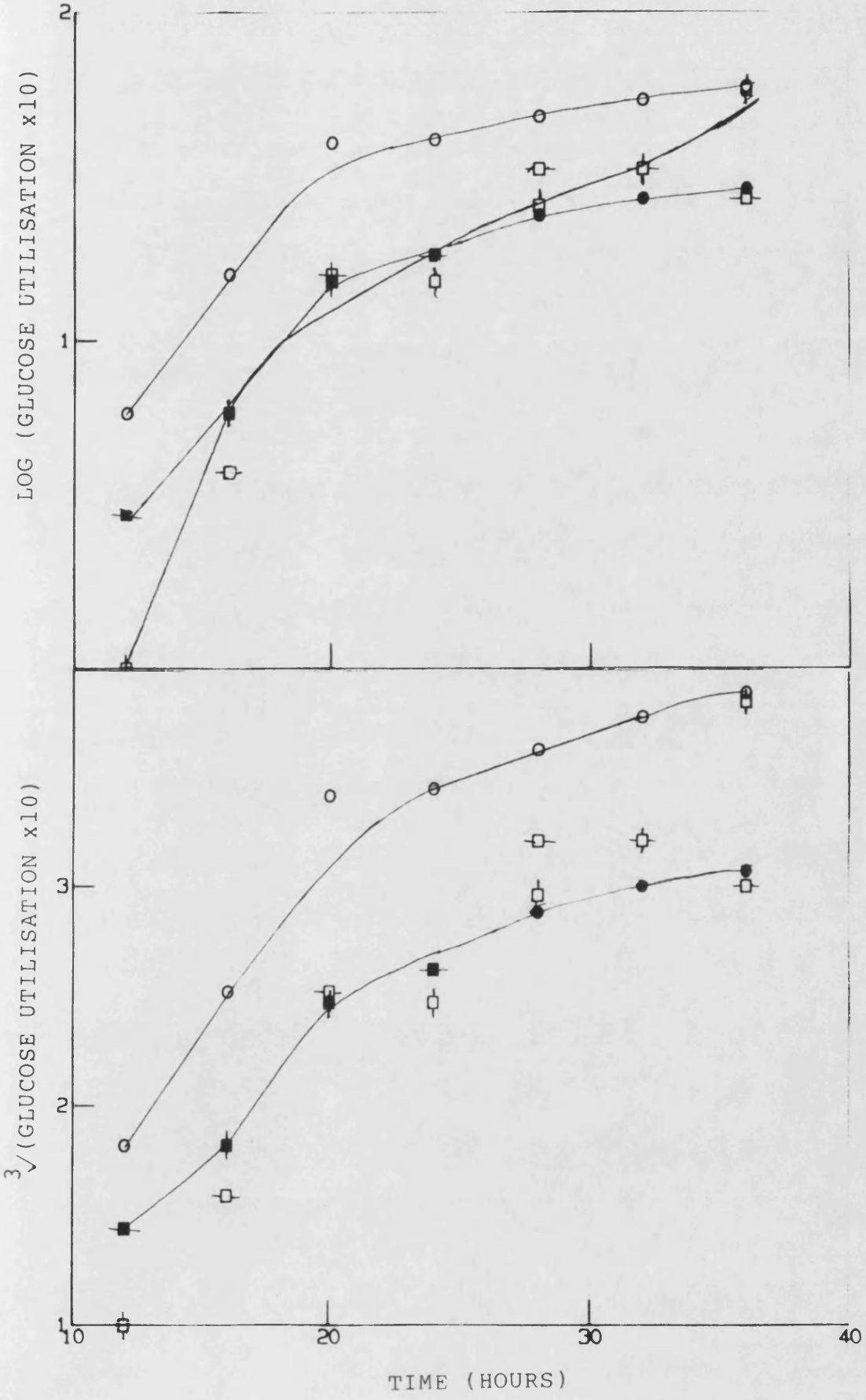

LOG (GLUCOSE UTILISATION x10)
2
1
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
3
2
1
10
20
30
40
TIME (HOURS)

FIGURE 3.18

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 6 $x10^3$ spores ml^{-1} (Expt IE2). Flask 9 (o), flask 10 (●), flask 11 (□), flask 12 (■).

FIGURE 3.19

Log of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 6 $x10^3$ spores ml^{-1} (Expt IE2). Flask 9 (o), flask 10 (●), flask 11 (□), flask 12 (■).

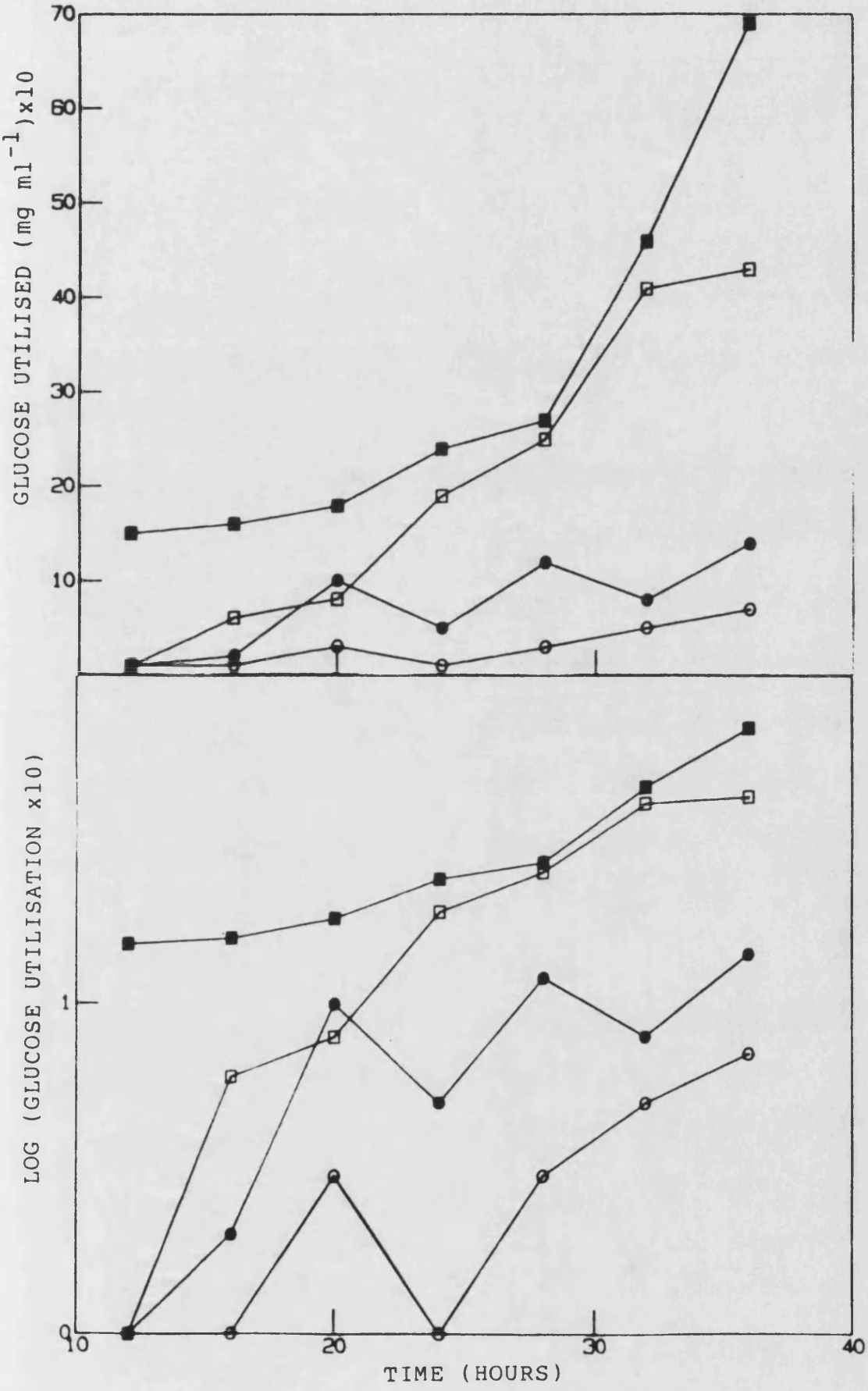

GLUCOSE UTILISED (mg ml^{-1})x10
70
60
50
40
30
20
10
LOG (GLUCOSE UTILISATION x10)
1
0
10
20
30
40
TIME (HOURS)

FIGURE 3.20

Cube root of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 6 $x10^3$ spores ml^{-1} (Expt IE2). Flask 9 (○), flask 10 (○), flask 11 (□), flask 12 (■).

FIGURE 3.21

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 3 $x10^3$ spores ml^{-1}. Tween 80 was present at a concentration of 0.002% (Expt IE2). Flask 13 (○), flask 14 (●), flask 15 (⊟), flask 16 (⏀).

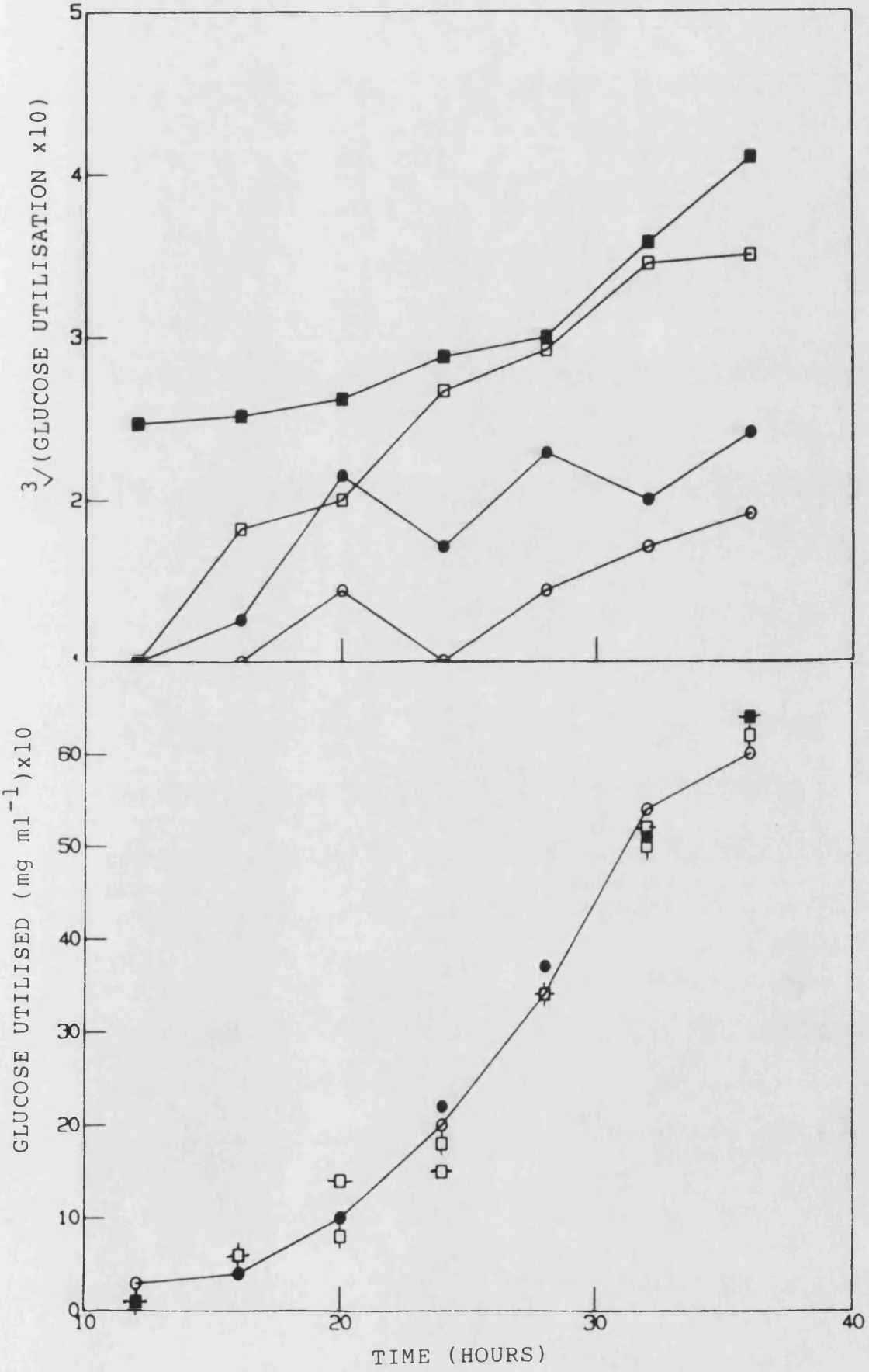
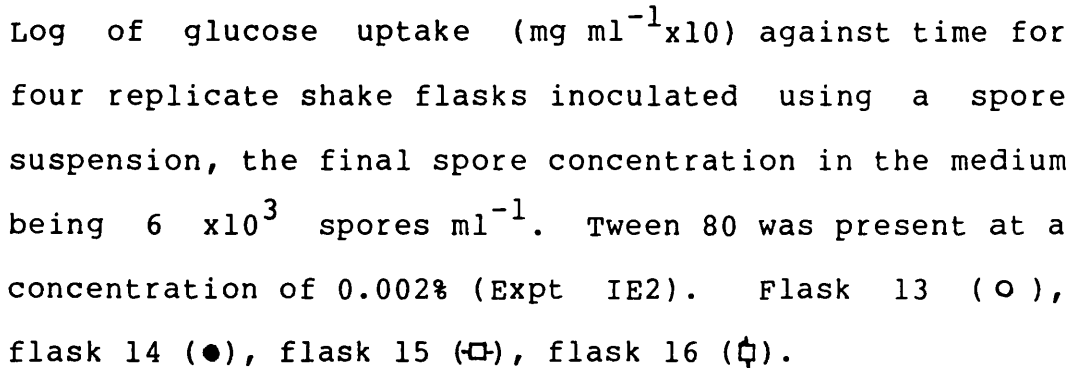
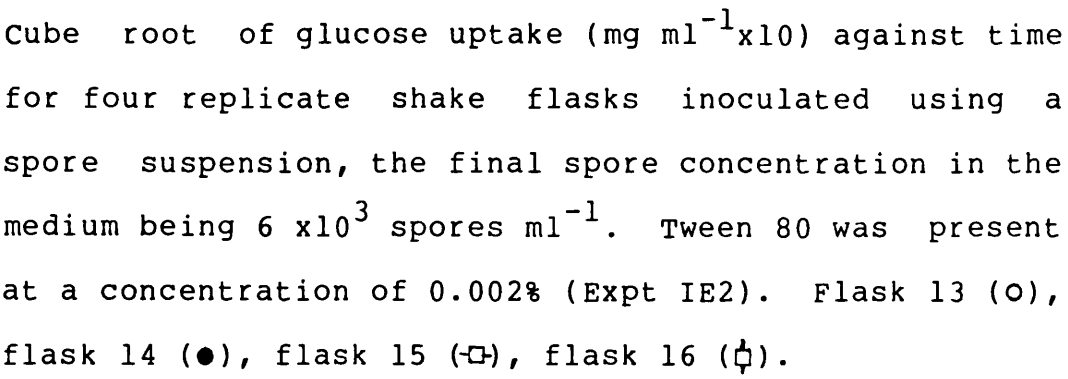

$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
5
4
3
2
GLUCOSE UTILISED (mg ml^{-1})x10
60
50
40
30
20
10
0
10
20
30
40
TIME (HOURS)

FIGURE 3.22



Log of glucose uptake (mg ml^{-1}x10) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 6 $x10^3$ spores ml^{-1}. Tween 80 was present at a concentration of 0.002% (Expt IE2). Flask 13 (o), flask 14 (●), flask 15 (⊡), flask 16 (⏀).

FIGURE 3.23



Cube root of glucose uptake (mg ml^{-1}x10) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 6 $x10^3$ spores ml^{-1}. Tween 80 was present at a concentration of 0.002% (Expt IE2). Flask 13 (o), flask 14 (●), flask 15 (⊡), flask 16 (⏀).

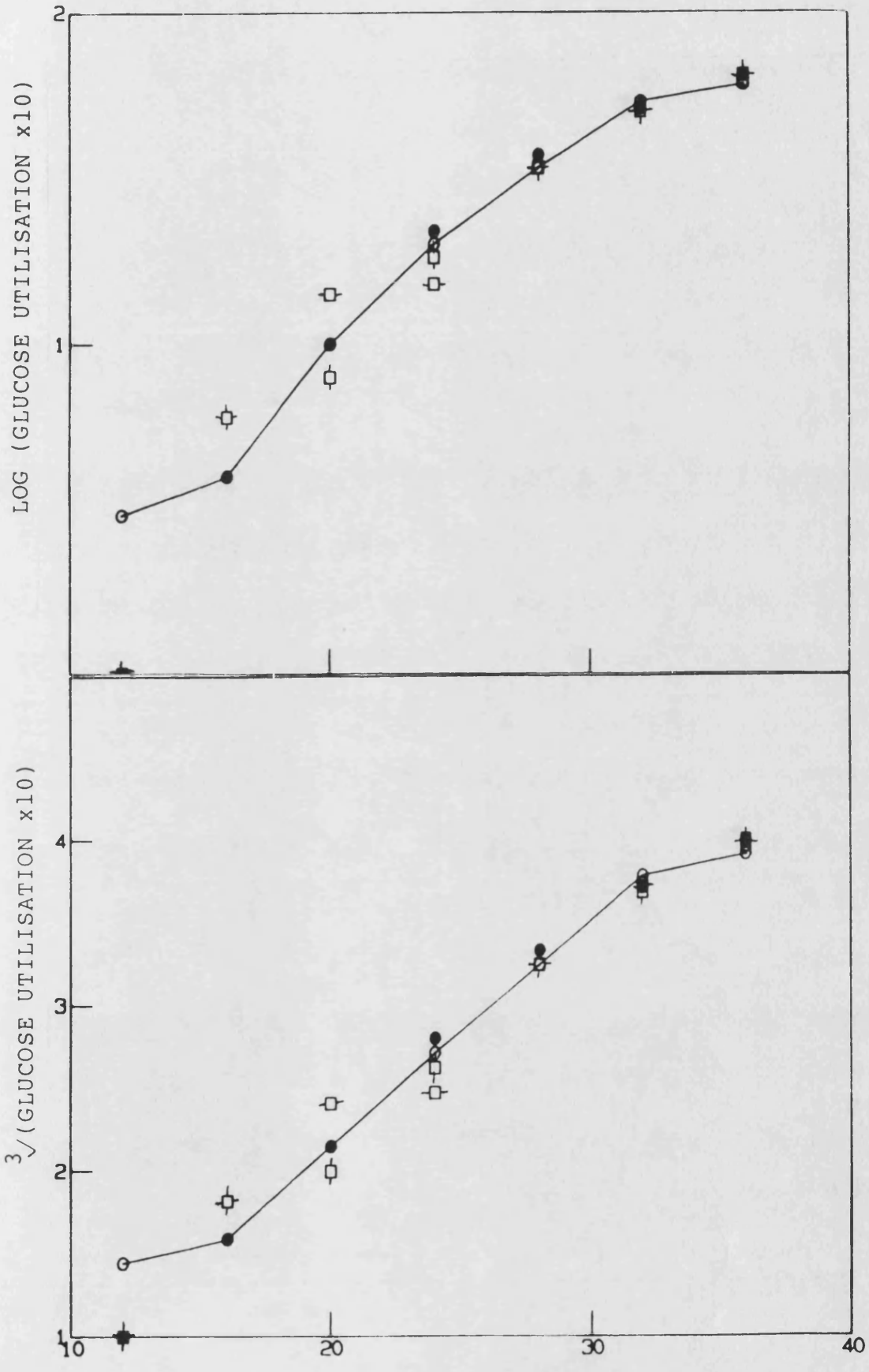

LOG (GLUCOSE UTILISATION x10)
2
1
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
4
3
2
1
10
20
30
40
TIME (HOURS)

FIGURE 3.24

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a slope inoculum (Expt IE2). Flask 17 (○), flask 18 (●), flask 19 (□), flask 20 (■).

FIGURE 3.25

Log of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a slope inoculum (Expt IE2). Flask 17 (○), flask 18 (●), flask 19 (◰), flask 20 (■).

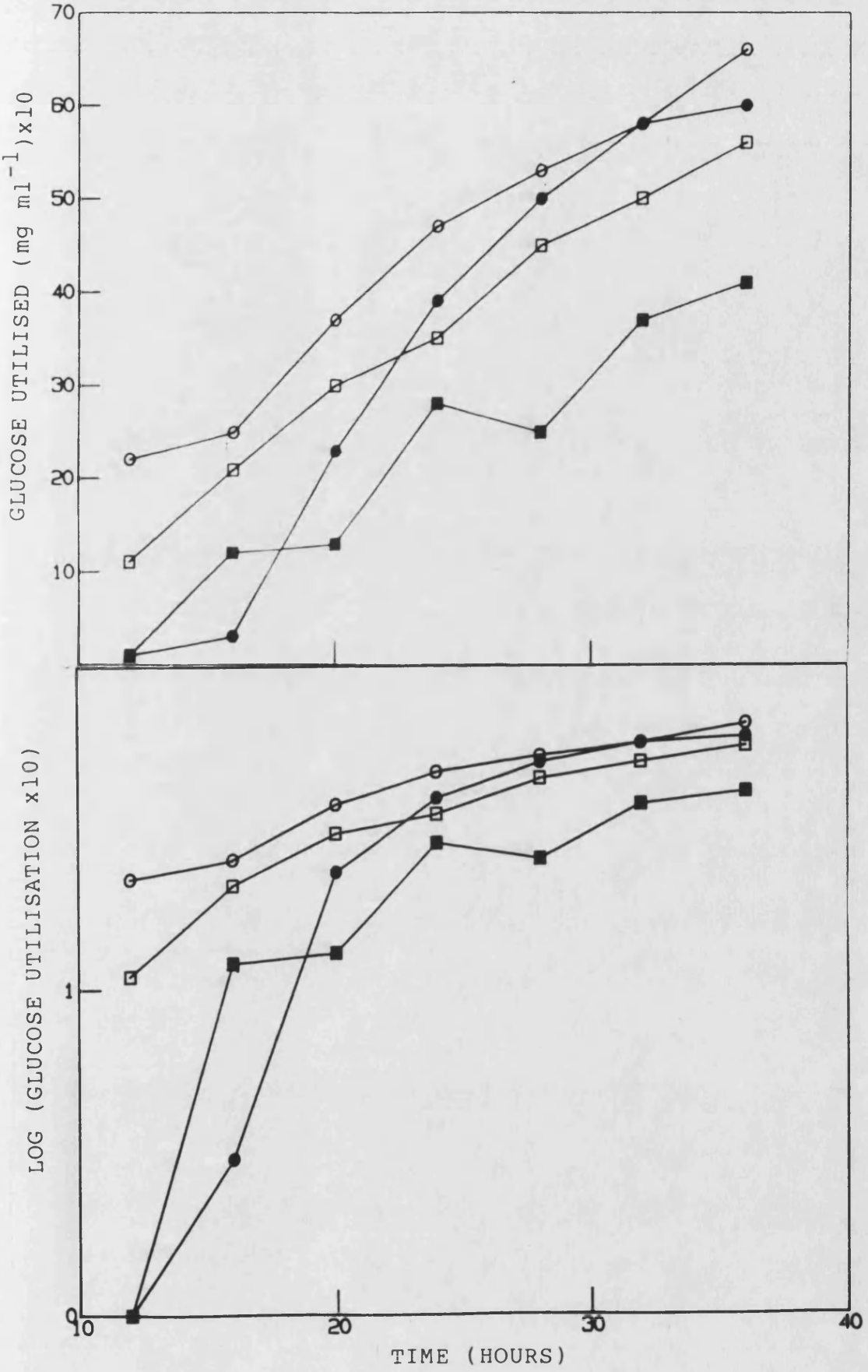
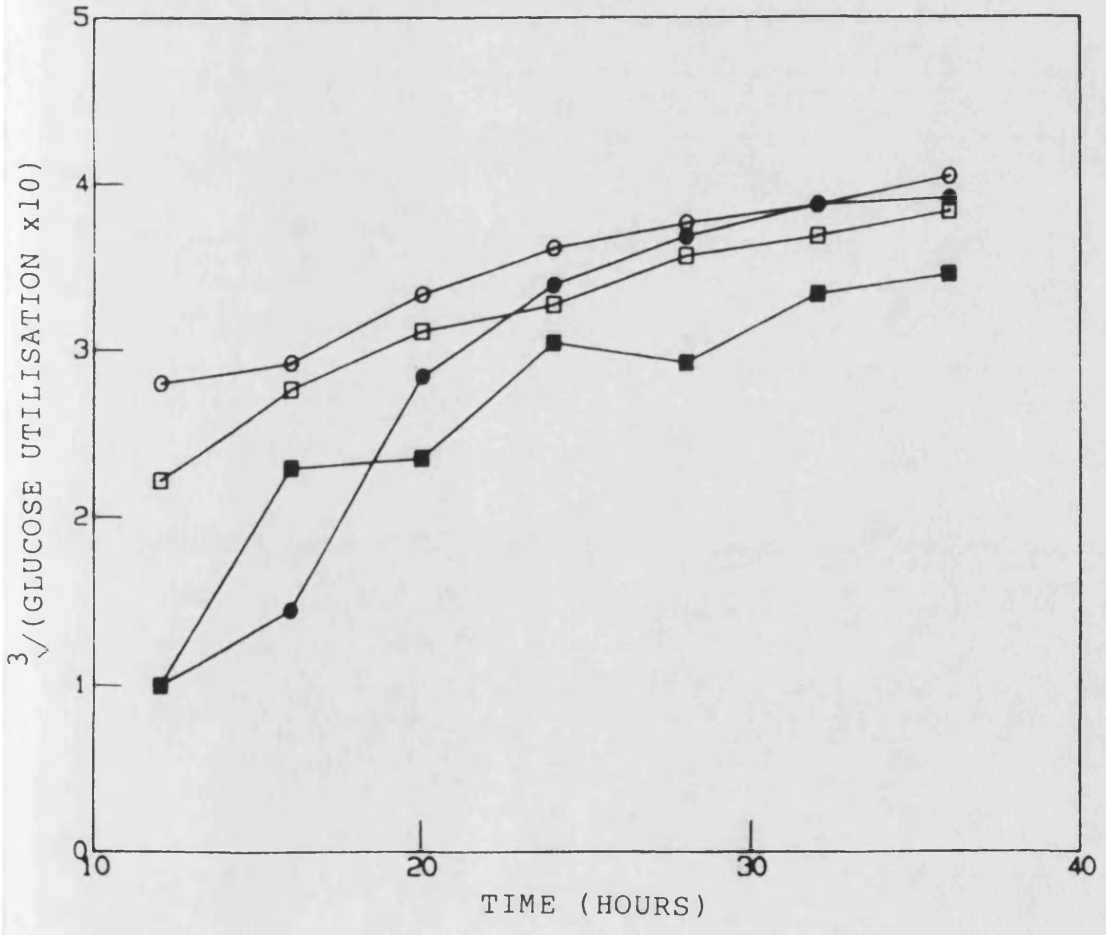

GLUCOSE UTILISED (mg ml^{-1})x10
70
60
50
40
30
20
10
LOG (GLUCOSE UTILISATION x10)
1
0
10
20
30
40
TIME (HOURS)

FIGURE 3.26

Cube root of glucose uptake (mg ml^{-1}x10) against time for four replicate shake flasks inoculated using a slope inoculum (Expt IE2). Flask 17 (○), flask 18 (●), flask 19 (□), flask 20 (■).



Experiment IE3

The speed of glucose uptake in these flasks varied greatly and it was not possible to sample the flasks frequently enough. As a result a maximum of 5 points per graph were plotted (figs 3.27-3.38). This did not allow much useful kinetic data to be extracted from the growth curves.

All but one of the flasks inoculated with mycelial mats and agar exhibited mycelial growth. Generally 20% of glucose was utilised in the first 12 hours of the fermentation. The lag phase, if there was one, was not detectable and most of the glucose in the medium was exhausted after 28 hours (figs 3.27-3.29).

Because of the complexity of the culture conditions there may well have been several limiting factors acting on the cultures and logarithmic and cube root growth curves were not calculated. The inhibiting factors could include toxic metabolites stored in the agar or mycelia, inhibition of spore germination by metabolites and oxygen limitation caused by the agar and high potentially metabolising concentration of mycelial mat. These inhibitions could explain why the three sets of replicates inoculated with spores, mycelia and agar showed such similar growth characteristics. There were however, large differences between glucose concentrations between the flasks both at the beginning and end of the fermentation monitoring period. The variation between curves could be as much as 3 mg ml^{-1} after 28 hours of

fermentation (fig 3.27).

This was in marked contrast to the flasks inoculated with spores which exhibited pelleted growth. These flasks all utilised 10% or less of the medium glucose after 12 hours. After 12 hours the growth became rapid and in some cases followed cube root kinetics (eg fig 3.27). Additives such as tween 80 and antifoam B increased the growth rate of the cultures considerably (tables 3.19 to 3.21). The higher tween 80 concentration used in experiment IE3 did not have as great an effect on standardising growth as in experiment IE2.

The use of ten day old spores in this experiment did not give very different results to the six day old spores used in experiment IE2.

FIGURE 3.27

Glucose uptake (mg ml^{-1}x10) against time for four replicate shake flasks inoculated using a quarter of a petri dish culture as an inoculum (Expt IE3). Flask 1 (○), flask 2 (●), flask 3 (□), flask 4 (■).

FIGURE 3.28

Glucose uptake (mg ml^{-1}x10) against time for four replicate shake flasks inoculated using a half of a petri dish culture as an inoculum (Expt IE3). Flask 5 (○), flask 6 (●), flask 7 (□), flask 8 (■).

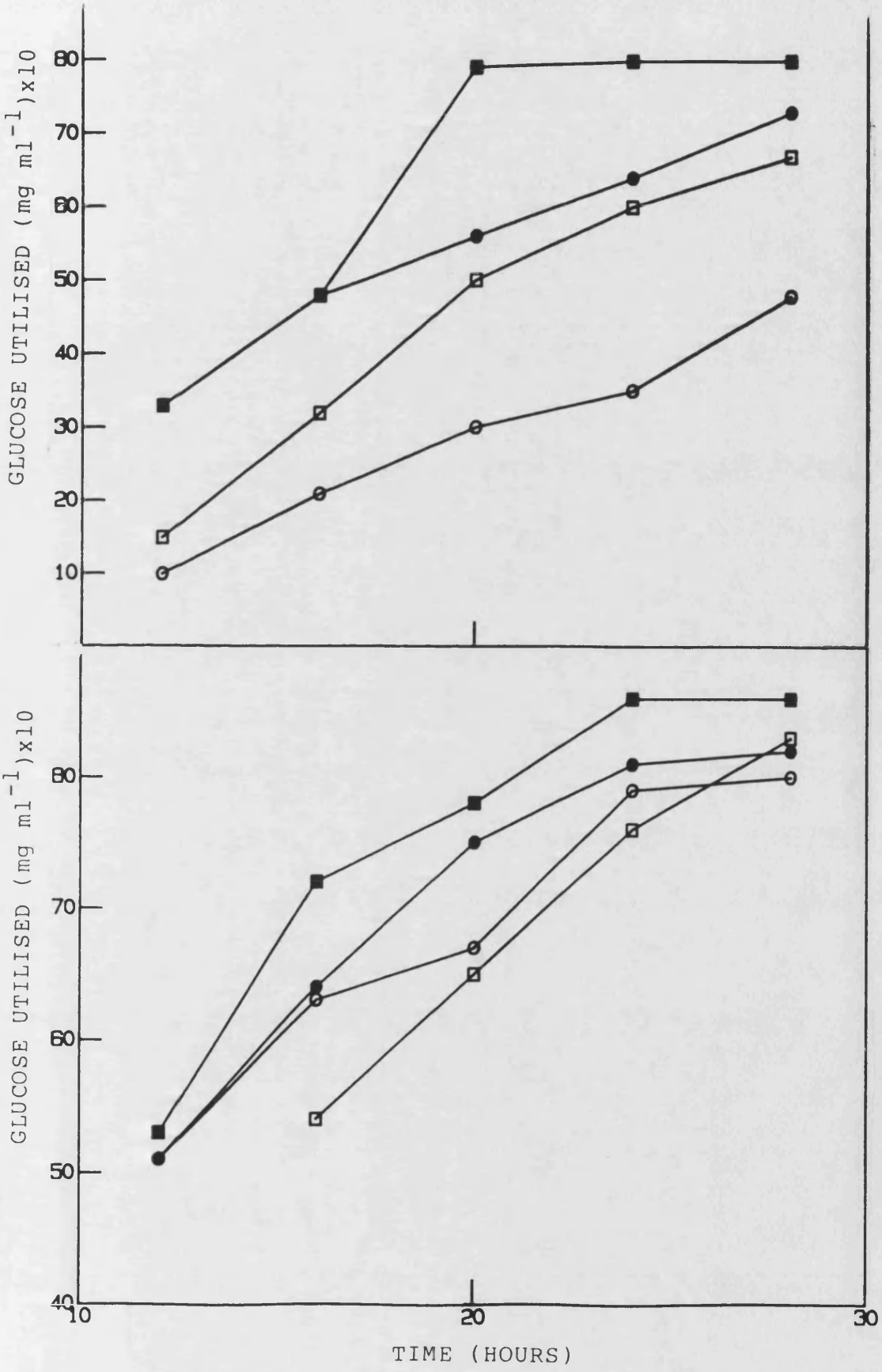

GLUCOSE UTILISED (mg ml^{-1})x10
80
70
60
50
40
30
20
10
GLUCOSE UTILISED (mg ml^{-1})x10
80
70
60
50
40
10
20
30
TIME (HOURS)

FIGURE 3.29

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using an eigth of a petri dish culture as an inoculum (Expt IE3). Flask 9 (◘), flask 10 (●), flask 11 (□), flask 12 (■).

FIGURE 3.30

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 3.7×10^4 spores ml^{-1} (Expt IE3). Flask 13 (o), flask 14 (●), flask 15 (□), flask 16 (■).

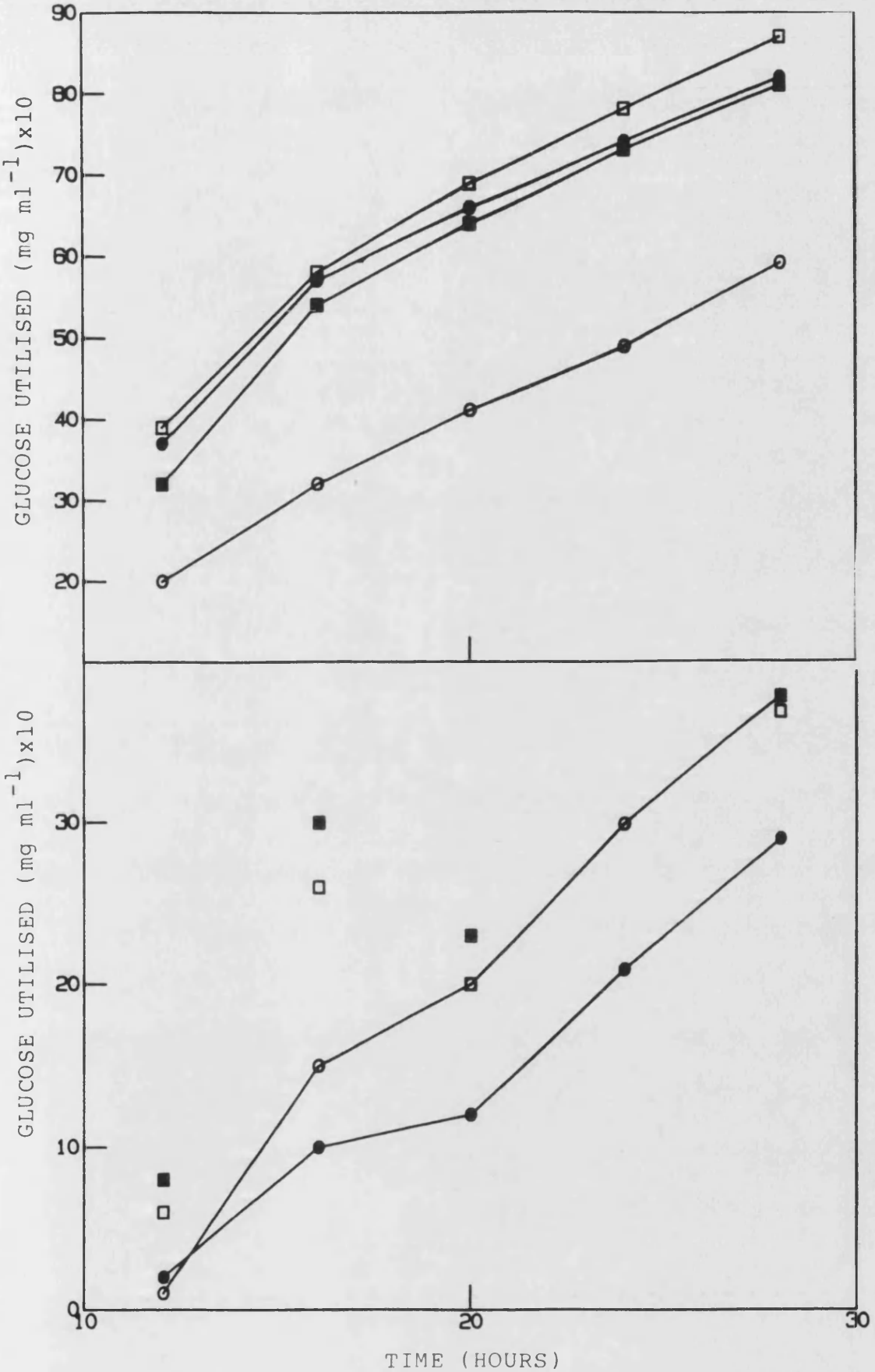

GLUCOSE UTILISED (mg ml^{-1})x10
90
80
70
60
50
40
30
20
GLUCOSE UTILISED (mg ml^{-1})x10
30
20
10
0
10
20
30
TIME (HOURS)

FIGURE 3.31

Log of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 3.7 $x10^4$ spores ml^{-1} (Expt IE3). Flask 13 (○), flask 14 (●), flask 15 (□), flask 16 (■).

FIGURE 3.32

Cube root of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 3.7 $x10^4$ spores ml^{-1} (Expt IE3). Flask 13 (○), flask 14 (●), flask 15 (□), flask 16 (■).

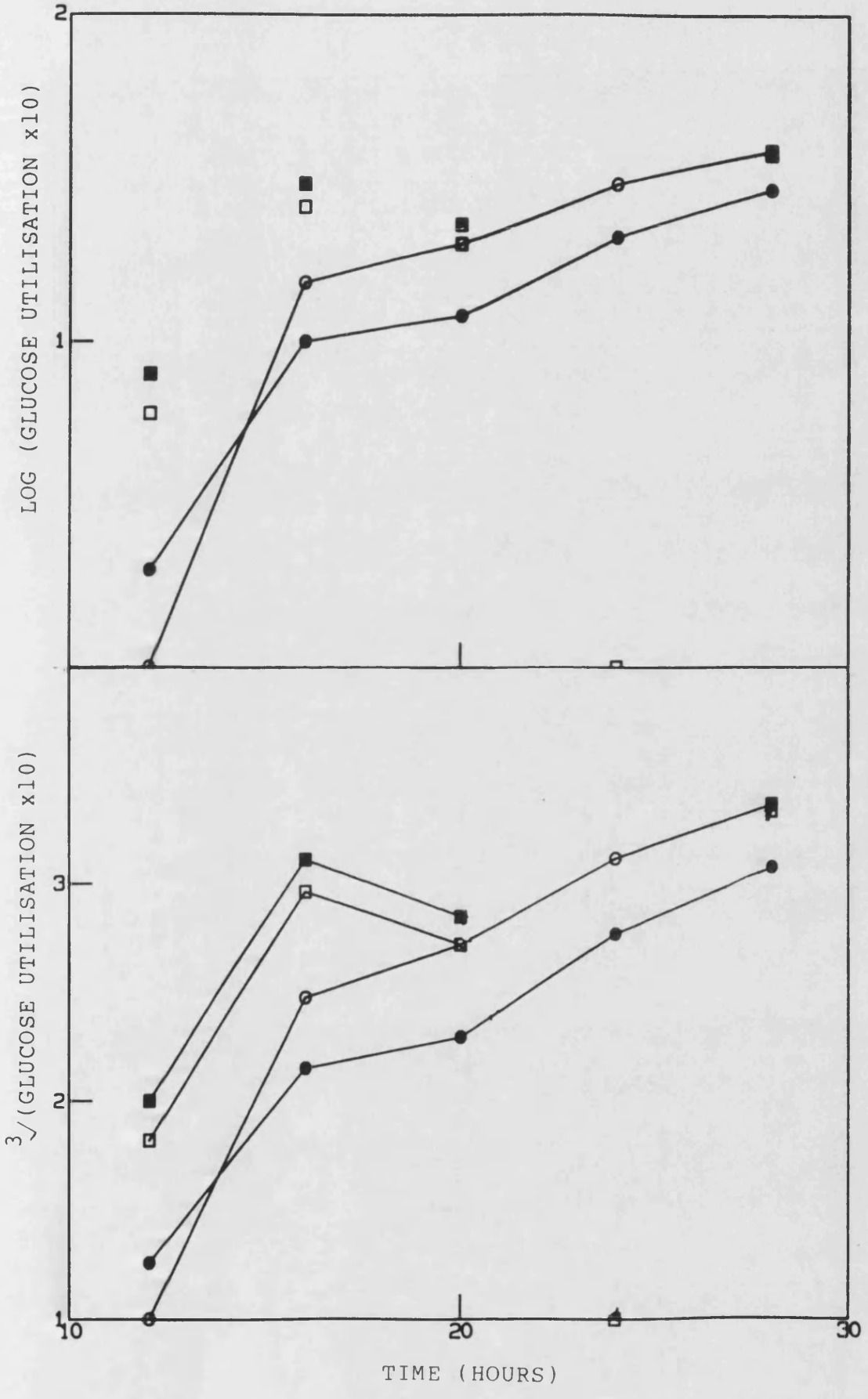

LOG (GLUCOSE UTILISATION x10)
2
1
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
3
2
1
10
20
30
TIME (HOURS)

FIGURE 3.33

Glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 3.7×10^4 spores ml^{-1}. Antifoam B was present at a concentration of 100 ppm (Expt IE3). Flask 17 (o), flask 18 (●), flask 19 (□), flask 20 (■).

FIGURE 3.34

Log of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 3.7×10^4 spores ml^{-1}. Antifoam B was present at a concentration of 100 ppm (Expt IE3). Flask 17 (o), flask 18 (●), flask 19 (□), flask 20 (■).

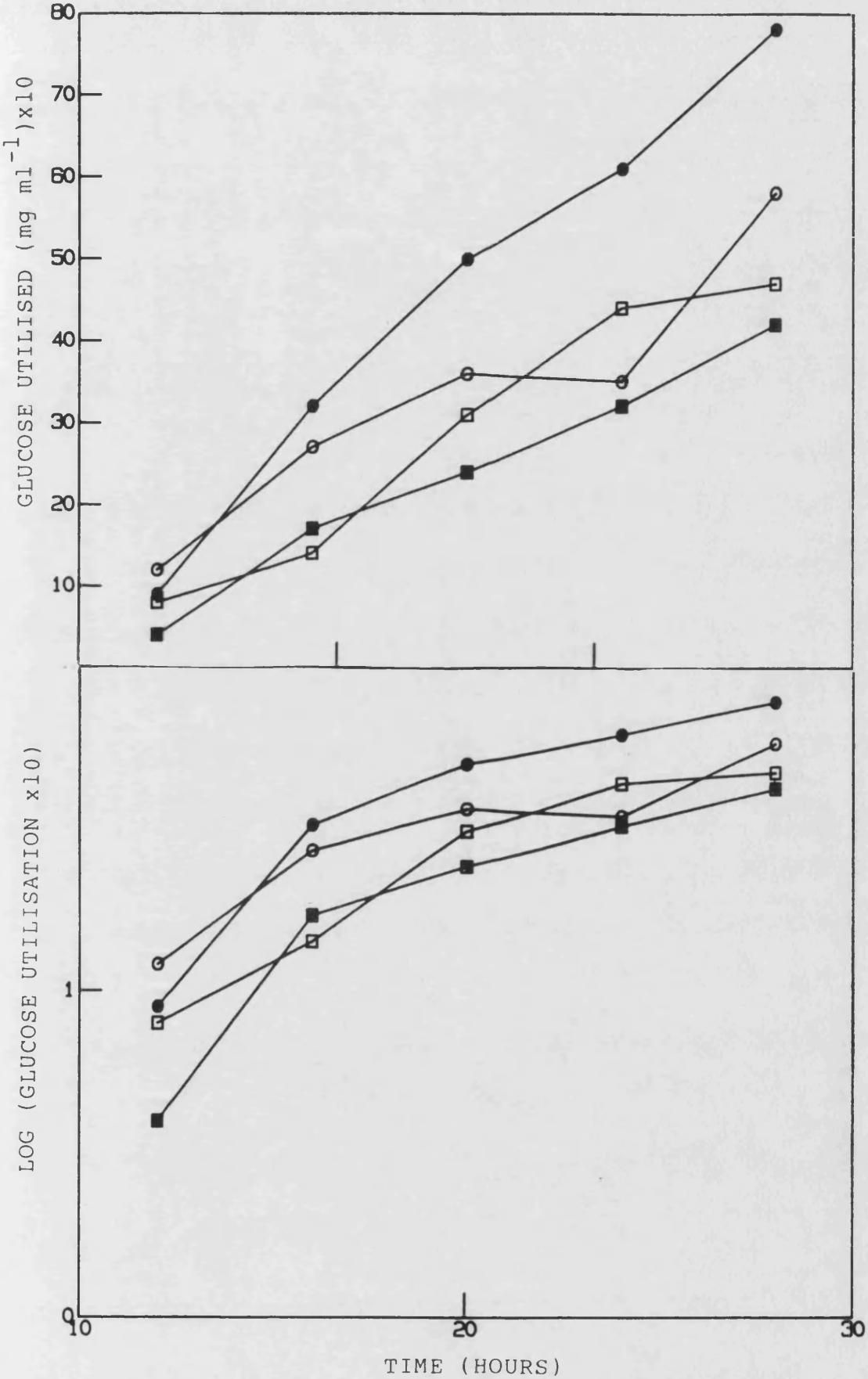

GLUCOSE UTILISED (mg ml^{-1})x10
80
70
60
50
40
30
20
10
LOG (GLUCOSE UTILISATION x10)
1
0
10
20
30
TIME (HOURS)

FIGURE 3.35

Cube root of glucose uptake (mg ml^{-1}x10) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 3.4 $x10^4$ spores ml^{-1}. Antifoam was present at a concentration of 100 ppm (Expt IE3). Flask 17 (o), flask 18 (●), flask 19 (□), flask 20 (■).

FIGURE 3.36

Glucose uptake (mg ml^{-1}x10) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 3.7 $x10^4$ spores ml^{-1}. Tween 80 was present at a concentration of 0.1% (Expt IE3). Flask 21 (o), flask 22 (●), flask 23 (□), flask 24 (■).

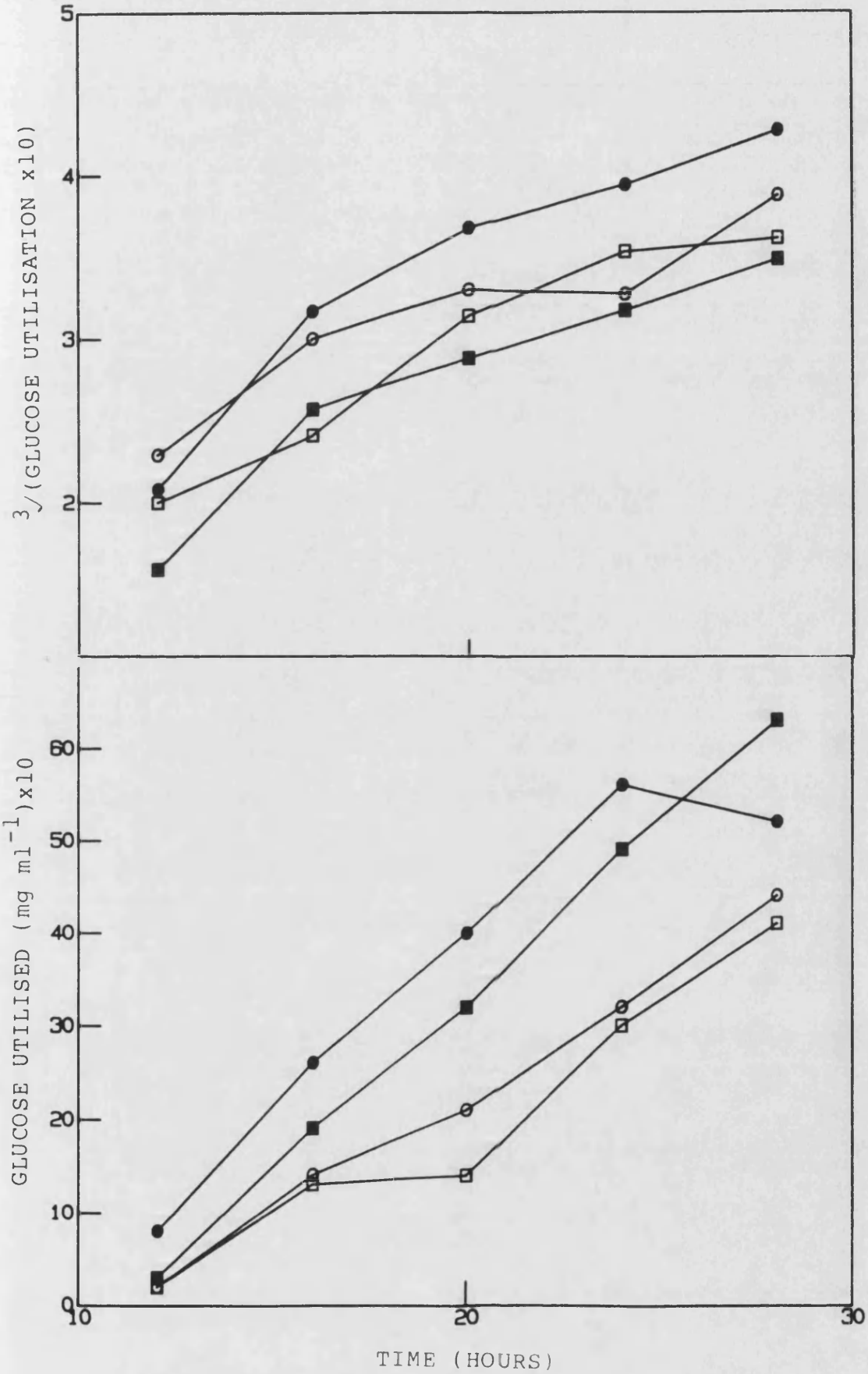
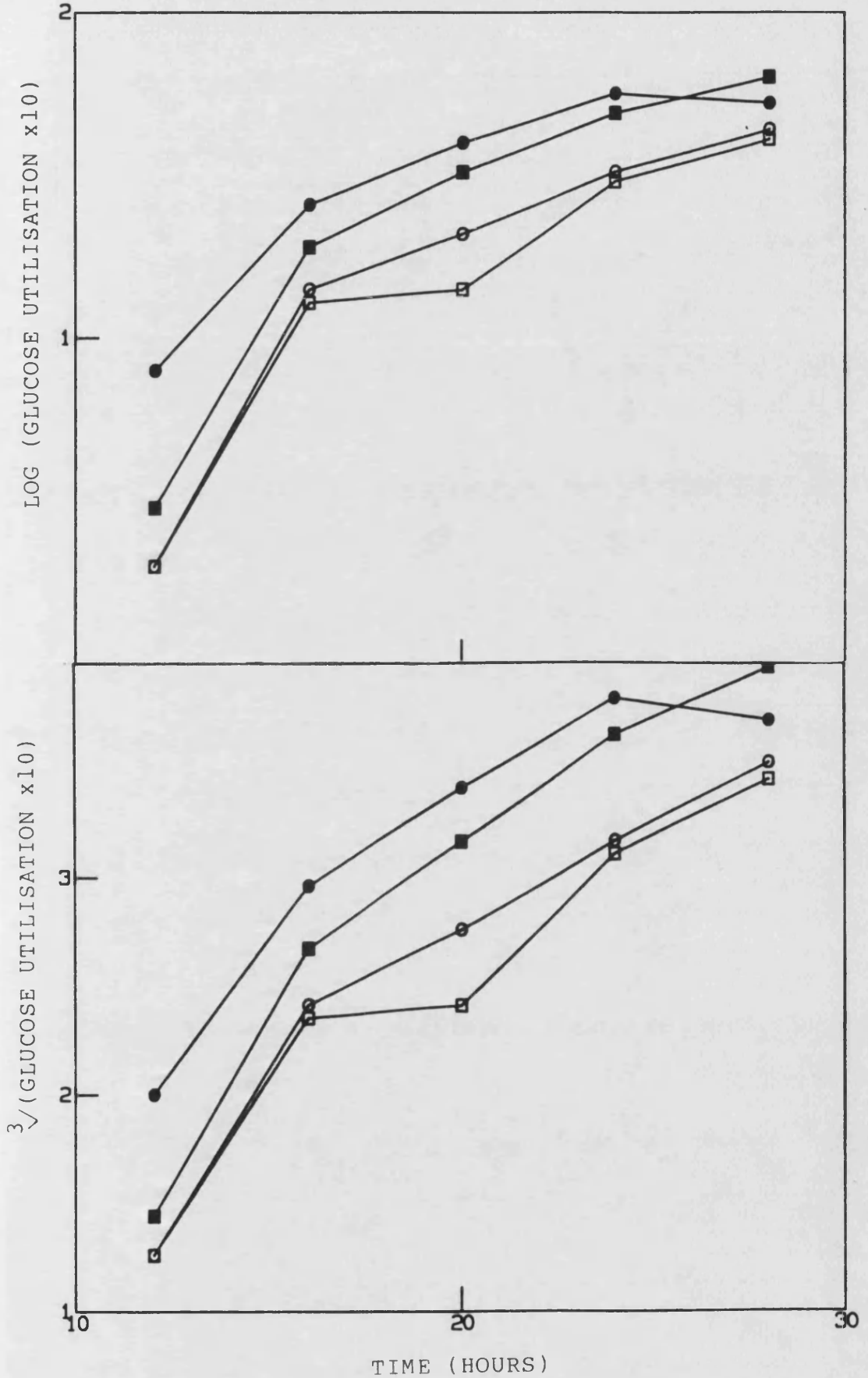

5
4
3
2
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
60
50
40
30
20
10
0
GLUCOSE UTILISED (mg ml^{-1})x10
10
20
30
TIME (HOURS)

FIGURE 3.37

Log of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 3.7 $x10^4$ spores ml^{-1}. Tween 80 was present at a concentration of 0.1% (Expt IE3). Flask 21 (○), flask 22 (●), flask 23 (□), flask 24 (■).

FIGURE 3.38

Cube root of glucose uptake (mg $ml^{-1}x10$) against time for four replicate shake flasks inoculated using a spore suspension, the final spore concentration in the medium being 6 $x10^3$ spores ml^{-1}. Tween 80 was present at a concentration of 0.1% (Expt IE3). Flask 21 (○), flask 22 (●), flask 23 (□), flask 24 (■).


LOG (GLUCOSE UTILISATION x10)
2
1
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
3
2
1
10
20
30
TIME (HOURS)

3.4 Discussion of Shake Flask Experiments

C.bainieri assumed the three modes of growth referred to in chapter 1 when grown in shake flasks. Pellets, together with an attached ring in the same flask, were the most common form of growth. Filamentous growth only occurred in a limited range of conditions such as large spore concentrations coinciding with the presence of exhausted agar. Flasks containing filamentous growth also exhibited ring growth. The only flasks not exhibiting heavy growth of this type were the flasks treated with tween 80.

Increasing the spore concentration in shake flasks did not have the desired effect of inducing mycelial growth. The effect on glucose uptake was not easy to determine because of the variations in size of the pellets formed.

The problem in interpreting the shake flask results lay in the different growth forms which were exhibited by the fungus. Pellet size distribution was almost certainly the root of the problem. If a strain of fungus is grown in shake flasks and one flask contains pellets of 1 cm diameter and another contains an equal mass of 1mm diameter pellets, assuming all other properties are identical, the small pellets will grow more rapidly than the large ones. Papers assessing the effects of pelleted growth on the rheology of culture media always use pellets in the size range of <1mm. The more densly packed the mycelia within the pellets the

more slowly diffusion will occur into the pellets. Pellet size and mycelial density were factors that could not be easily investigated. Given more time and an image analyser the relationship between different inoculation and growth regimes and pellet size could have been investigated.

The potential complexity of the fungal pellet has already been discussed in chapter 1. Differentiation within the pellets, toxic metabolites diffusing in and out of the pellet and nutrients diffusing inwards could all provide a range of environments within a single pellet. Consequently the best that can be achieved with large pellets is to optimise the conditions for drug conversion by pellets of any particular size.

Despite the inability to determine the growth form or the size of pellets of the fungus it was still possible to use shake flask cultures to determine the effects of antifoams B and C, Tween 80 and spore concentration on C.bainieri.

Once formed the pellets of C.bainieri were never seen to fragment. Combined with the inability to grow C.bainieri reliably and repeatedly as a filamentous organism in defined media this indicated that chemostat culture would be difficult to achieve. Chemostats will only work with organisms which divide by some means and the growth forms obtained at this time were not suitable for such division. The shake flask experiments showed that the use of spore inocula for the culture of

C.bainieri was possible and that similar results to slope inocula could be produced. This was important as it showed that inoculation of fermenters with high concentrations of spores was possible. Reduction of the variability between replicates caused by adding antifoam or tween 80 was important. Foaming produced by tween 80 in shake flasks would have led to unacceptable levels of foaming in stirred vessels.

Variability was found to be high in most of these experiments. When the individual growth curves for the shake flasks were examined they provided much more information than pooled data. Approximations of μ max for the logarithmic phase of growth could be calculated from IE1. The calculated figures for samples 5 and 6 were μ max = 0.125 and 0.35 h^{-1} respectively (apendix 3). This is within the known range for fungi (99,122).

CHAPTER 3

Summary

A technique for the production and harvesting large numbers of C. bainieri spores has been developed. These spores can be used to inoculate several fermenters (Sect 3.2.3) and large numbers of shake flasks simultaneously (Sect 3.3.4).

When used in the presence of tween 80 (0.02%), a dense rapidly growing culture of small pellets was produced. These pellets produced very similar growth curves in each of the shake flasks, they were cultured in (Figs 3.21 to 3.23, pp 120-123).

Mycelial cultures could be produced by adding sections of mycelial mat and spent agar to the shake flasks (p 127).

Three inoculum regimes for the fermenters were possible:

i) Direct inoculation with spores
ii) Inoculation with numerous small pellets
iii) Inculation with mycelia.

Direct inoculation with spores was the preferred method as it resulted in the addition of the smallest amount of extraneous material to the fermenters and the quantity of added biomass could be easily quantified by spore counts.

Norcodeine production occurs in glucose depleted cultures and peak norcodeine concentrations (approximately 12% conversion) were achieved 12 days post inoculation (glucose was completely

depleted after 5 days incubation (Table 3.6)). This suggests that codeine conversion is inhibited by glucose metabolism or active growth.

Investigation of codeine conversion should be carried out using:

(1) Continuous culture of C. bainieri growing in its filamentous form.

(2) Comparison of batch cultures of various types (Section 1.7). These should include:

i) Very small pellets - enhance the surface area available to the medium and reduce diffusional limitations.

ii) Drain and fill operations carried out by sedimenting biomass and replacing medium should be attempted to see if norcodeine production from a single batch of organisms can be increased.

Airlift Fermenter Design

4.1 Introduction

At the time the airlift fermenter was built there was little theoretical data available on the design of airlift fermenters and even less available on the design of small airlift fermenters. As can be seen from the introduction the height, diameter and liquid volume of the main fermenter body have considerable effects on the efficiency of operation of airlift fermenters. The height of the draught tube above the fermenter bottom, diameter of the draught tube and the liquid height above the draught tube also have considerable relevance to the design of ALFs. These features, along with the growth form of C.bainieri set the initial design constraints. None of the more exotic types of airlift especially the hybrid designs (1,10,78) were considered for this project as they detract from the simplicity of the design.

4.2 The Effects of C.bainieri Growth on the Design of an Airlift Fermenter

Two factors were considered to be of great importance in ALF design, one was the size of the fungal pellets and the second was attached growth of the fungus. A third factor of concern was the possibility of foaming of the growth medium during culture.

4.2.1 Pellet Size

Shake flask cutures of C.bainieri had exhibited varying pellet sizes (up to 2 cm diameter). In low shear environments such as airlift fermenters (64) there was the possibility that pellets would have a larger diameter than in high shear environments such as STFs. This was believed to be a result of the lower physical stress on the hyphae (64). As the 2cm diameter pellets were rarely seen in shake flask cultures the provision of a 2cm gap at the narrowest part of the fermenter was considered to be sufficient.

4.2.2 Attached Growth

Possible attachment sites for fungi were reduced to a minimum. This was achieved by keeping the number of protrusions into the medium as small as possible and by ensuring that most of the fermenter was continually washed with medium, thus reducing the number of dead areas and limiting the possibility of micro organisms settling on surfaces.

4.2.3 Foaming

The maximum possible volume was allowed for gas disengagement so that low levels of foaming did not cause medium loss through the air exhaust condenser and filter. It was hoped that the downflow of medium from the disengagement area wold also help prevent problems caused by foaming by reducing the amount of foaming as

previously reported (52).

4.3 Physical Constraints on Fermenter Design

4.3.1 Working Volumes

The smallest efficient airlift designs reported were of approximately 4.5 liter working volume (fig 1.3).

4.3.2 Aspect ratio

An aspect ratio (height:diameter) of approximately 5:1 was considered the minimum efficient ratio (10). According to Moresi (90) the best mixing and oxygen transfer rates an larger internal loop airlift fermenters should occur at aspect ratios of 30:1.

4.3.3 Fermenter Diameter

Frictional resistance to liquid circulation was claimed by Hatch to be lowered if the fermenter was at least 30cm in diameter (52). Unfortunately because of other size restrictions the maximum diameter if the medium filled section of the fermenter was 10cm. The air disengagement section at the top of the fermenter was wider than the rest of the fermenter to reduce the height of the assembly and give an area of reduced turbulence to assist in the disengagement of the air.

4.3.4 Draught Tube Design

4.3.3.1 Diameter

Several workers (105) have found that the ratio between the draught tube diameters and fermenter diameter should be approximately 0.7. This was found to provide almost equal areas for upflow and downflow of liquid within the fermenter as shown below (table 4.1).

Table 4.1

THE RELATIONSHIP BETWEEN THE DIAMETERS OF FERMENTERS AND DRAUGHT TUBES AND THE AREAS AVAILABLE FOR LIQUID UPFLOW AND DOWNFLOW

fermenter diameter (cm) -	draught tube diameter (cm) -	total cross sectional area (cm^2) -	draught tube cross sectional area (cm^2)	annulus cross sectional area (cm^2)
10	6	78.5	28.3	50.2
10	7	78.5	39.5	39.0
15	10.5	176.7	86.6	90.1
20	14	314.2	153.9	160.3

Sections of QVF piping of 10 and 15 cm diameter were considered as a basis of the fermenter outer wall design. As the 10 cm diameter tube was selected the draught tube diameter was chosen to be as large as possible whilst still allowing a 2cm gap for the passage of fungal pellets. Therefore a 6 cm diameter draught tube was

used.

4.3.3.2 Height

The height of the draught tube was set at the point at which the disengagement space started (63 cm total). This was based on the observations of Hatch that the draught tube should be as long as possible without actually constricting the flow of the medium (52).

4.3.3.3 Height of Draught Tube Above Fermenter Bottom

In order to prevent the draught tube acting as a valve the height of the bottom of the draught tube from the fermenter base had to provide a gap large enough for the free flow of culture medium.

The cross sectional area of the annulus was 50.2 cm^2. As the area of the curved wall of a cylinder is the product of its height and circumference athe minimum height of the draught tube from the fermenter base was calculated.

height = 50.2/2pi r

=2.7cm

Initial experiments used a much larger gap than this.

4.4 Other Factors Affecting ALF Design

In addition to the constraints mentioned above various factors were taken into account, including size, materials, of construction, volume etc.

4.4.1 Size

The autoclaves available in the university were of a limited height and there were problems with the availability of steam for sterilisation outside an autoclave. Therefore the fermenter had to be robust and short enough to be transported. Originally the fermenter was designed with autoclaving in mind but alterations to the design which were necessary for sucessful construction increased the height of the fermenter so that it could not be autoclaved.

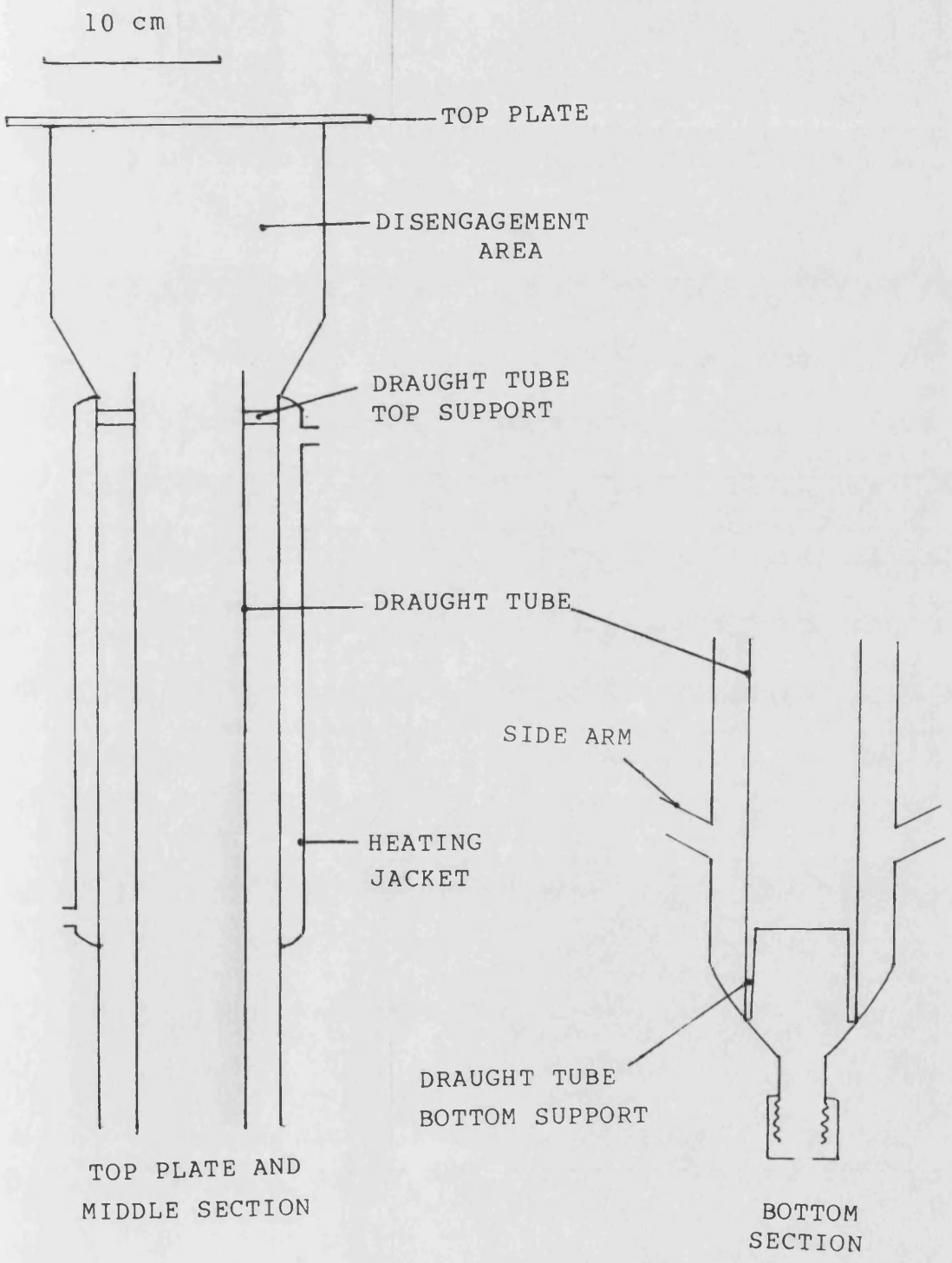
The height of the fermenter was increased because of the use of the flared end of QVF piping to join the sections of fermenter. These pipe ends had to be at least 10 cm long because shorter lengths were very difficult to cut and join onto other pipe sections.

4.4.2 Materials

Glass was used for the construction of the majority of the fermenter. This allowed easy observation of the fermentation. Glass was also comparitively cheap, easily avaliable, easily worked and modified and standard pieces such as side arms for sampling were avalable off the shelf. Welding facilities for stainless steel were not readily available and so wherever possible the use of stainless steel was avoided.

Figure 4.1

A Diagram of the Air Lift Fermenter. The Bar on the Diagram Represents Ten Centimetres.



Two of the effects of using glass for the construction of the fermenter were :-

i The fermenter was increased in height for reasons described above.

ii Metal supports for the draught tube would have been thinner and less fragile than the glass supports used. To help prevent breakage of the support legs at the bottom of the draught tube the legs were originally attached to a glass ring. This had to be dispensed with as a large dead space was created by the ring.

4.4.3 Medium Volume

One of the original aims of the project was to try and grow the fungus in continuous culture. It was therefore desirable to keep the fermenter volume as low as was practicable to allow as long as possible to elapse between the changeover from one nutrient reservoir to the next. It is also easier to deal with small volumes of medium unless specialised facilities for large scale liquid sterilisation and handling are available.

4.4.4 Fermenter Sections

The fermenter was made in four sections the top plate, a middle section containing the heating jacket, a bottom section and the draught tube (fig 4.1). This enabled the rapid replacement of any sections, the replacement of draught tubes and also enabled thorough cleaning of the fermenter to be performed.

4.4.5 Medium Height

Some method of maintaining liquid volume in the fermenter had to be found. Hatch (52) had found that the medium level in air lift fermenters was of great importance. Passing large volumes of dry air through a fermenter for a period of days will lead to a considerable loss of liquid volume. Changes in liquid volume can be prevented by heating the air to the same temperature as the fermenter contents and then saturating the air with water. A second method is to condense most of the water from the outlet air stream and allow the condensate to drip back into the fermenter. As the second method appeared to be simpler and had the advantage of preventing the outlet air filter becoming wet this was the solution used. Once the fermenter was constructed and the optimal medium volume estimated several condensers had to be tested before one capable of reducing water losses to acceptable levels was found.

4.5 Outline of ALF Experiments

It was already known that ALFs could be sucessfully used to grow pelleted forms of filamentous fungi (6). The ALF was constucted for the purpose of cultivating C.bainieri with the hope of using the fungus to N-dealkylate codeine within the fermenter. Alterations of the design of the spargers and draught tube were carried out during the project but the medium

composition was kept constant. Different inoculum techniques, PTFE spraying and the effect of antifoam were also examined in these experiments.

4.6 Airlift Fermenter Construction

4.6.1 Materials

4.6.1.1 Top Plate

Stainless steel s.s. 316 was obtained from Avery, Knight and Bowen (Bath). Silicone rubber compound was obtained from RS electronics limited.

4.6.1.2 Glass Sections

Glass piping, fittings and side arms were obtained from QVF limited.

4.6.2 Methods

4.6.2.1 Top Plate

A 20 cm diameter 1/4" thick disc was cut from stainless steel plate (ss 316). Six lcm diameter bolt holes were drilled equidistant from each other and lcm from the edge of the plate.

Four equidistant ports were cut with their centres 5cm from the centre of the plate. A fifth port was cut in the centre of the plate.

Free machining stainless steel rod type EN 58 AM was used to make all the fittings for the fermenter top. Fittings for the holes were made from 1 1/2" diameter

rod. A second fitting (female stericonnector) was made from 1 1/8" diameter rod was screwed into this and the male stericonnector or blanks were made from 3/4"diameter rod. Silicone rubber compound was used to seal the joints between the top plates and ports (figs 4.2, 4.3).

4.6.2.2 Glass Section

a Middle Section

The top end of the section was made of the first 12cm of a 15cm i.d. QVF pipe section. This was connected to an 8cm long reducing section which was connected to a 10 cm long section taken from the end of a 10 cm i.d. QVF pipe (fig 4.1).

b Bottom section

A 10cm long 10cm i.d. pipe end was used to connect the bottom section to the middle section of the airlift fermenter. Below this section was a 8cm long 10cm i.d. pipe with six equally spaced 2cm diameter screw ended pipes for probes. Five centimetres down from the bottom of this section the fermenter narrowed to 2 cm diameter and a final 2 cm diameter screw ended glass pipe.

4.6.2.3 Draught tube

The draught tube was always a total height of 63 cm and 6 cm internal diameter.

The top of the draught tube was supported by

three 1cm long glass rods which were tipped with silicon rubber compound. Silicon rubber tubing with 6mm internal diameter was used to coat the support to help provide a smooth surface.

Three 0.5cm diameter glass rods were used as legs to support the bottom of the tubes. There were either 10 or 5 cm long and could be either attached to a 0.5 cm glass rod or not.

Early versions of the draught tube had the side supports placed 2cm from the top of the tube. In later versions the side supports were placed 1cm from the top of the tube.This was a position where the distance between the draught tube and the side and the fermenter was greatest thus reducing the blockage caused by attached growth on the supports.

4.6.2.4 Air Spargers

Three air spargers were used with the ALF for the culture of this fungus. These sparges were;

i) A 3cm diameter coarse glass sinter.

ii) A single oriface glass tube (0.2 cm internal diameter)

iii) A four orifice 'spider' with 0.1cm internal diameter tubing.

4.6.2.5 Airlift Fermenter Experiments

The various equipment described here was used to try and produce reproducible pelleted growth in the ALF.

Different combinations of inoculum type,sparger type and draught tube type were tried (chapt5)

The culture medium, temperature (27^{o}C), air flow rate (5l min^{-1})and initial pH value (5.1) were all kept constant.

If biological material was to be resuspended this was achieved by increasing the air flow rate to 10 l min^{-1}.

Codeine was added after 1 day of fermentation.

4.7 Airlift Control and Monitoring Development

Once the basic airlift fermenter had been developed the various control systems had to be tried and tested.

4.7.1 Temperature Control

4.7.1.1 Cooling

The air sparged into the fermenter produced a cooling effect, the temperature of the fermenter contents being about 2^{o}C lower than the ambient temperature in the abscence of heating. As the growth of C.bainieri was known to be low and the laboratory temperature was normally approximately 20^{o}C no other cooling was considered necessary.

4.7.1.2 Heating

Several heating methods including heating tape,

heating fingers, heated water jackets and a hot air cabinet were considered. Experiment showed that a hot water jacket could be used to maintain a constant 27°C temperature using warer circulated from a constant temperature tank of water maintained at 29°C.

4.7.2 Condenser Choice

A condenser was needed to prevent excessive water loss from the fermenter and also to prevent the exit of gas filter from becoming wet.

This part of the fermenter was very important because of the sensitivity of airlift systems to changes in liquid volume. The effectiveness of the condensers had to be tested rather than calculated. This was to ensure that condensate could return to the fermenter rather than be ejected as water droplets. Four different condensers were tested (table 4.2).

4.7.2.1 Method

The fermenter was filled with distilled water to thee 5.5l mark and compressed air at 15 psig was sparged through a glass sinter (3cm diameter,flat,coarse) and heated to 27°C. Condensers were left working for 24 hours and the rate of water loss calculated. Condensers were attaced to the fermenter as shown in figures 4.2 and 4.3.

TABLE 4.2

CONDENSERS USED TO PREVENT THE EVAPORATION OF WATER FROM THE AIRLIFT FERMENTER

Condensers

Condenser 1
Straight joint
Total length = 285 cm
Jacket length = 85 cm
Inlet/outlet diameter = 0.5 cm

Condenser 2 Liebig
Quickfit 19/26 neck and bottom joint

Condenser 3 Graham
Quickfit 19/26 joints
Internal coils - 20 cm long

Condenser 4
Quickfit 19/26 joints
Davis double surface condenser 15 cm long

Figure 4.2

The attachment of Condenser 1 to the ALF.

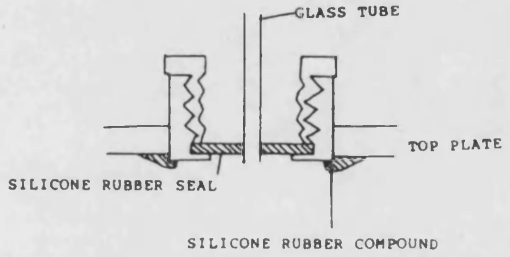
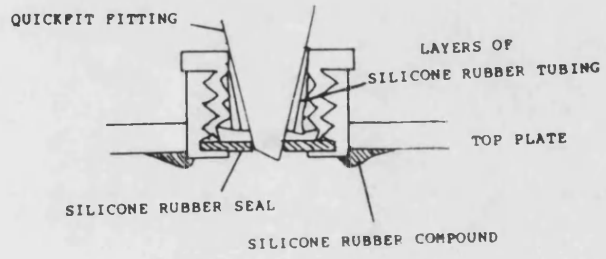


Figure 4.3

The Attachment of Condensers 2-4 to the ALF.



4.7.2.3 Chilled water

Cooling water for the condenser was supplied as on 80 % water and 20% antifreeze mixture. A cooling coil was used to control the temperature at 1^{o}C and a centrifugal pump was used to circulate the fluid. The water flow rate to the condenser was controlled using a needle valve.

4.7.2.4 Condenser tests

a) Condenser 1

This condenser was considered briefly but the narrow connecting tube proved to be a nuisance. Condensate could not flow freely back into the ALF and accumulated on the side of the connecting tube until the volume of liquid was large enough for droplets to be carried out of the condenser in the exit gas stream.

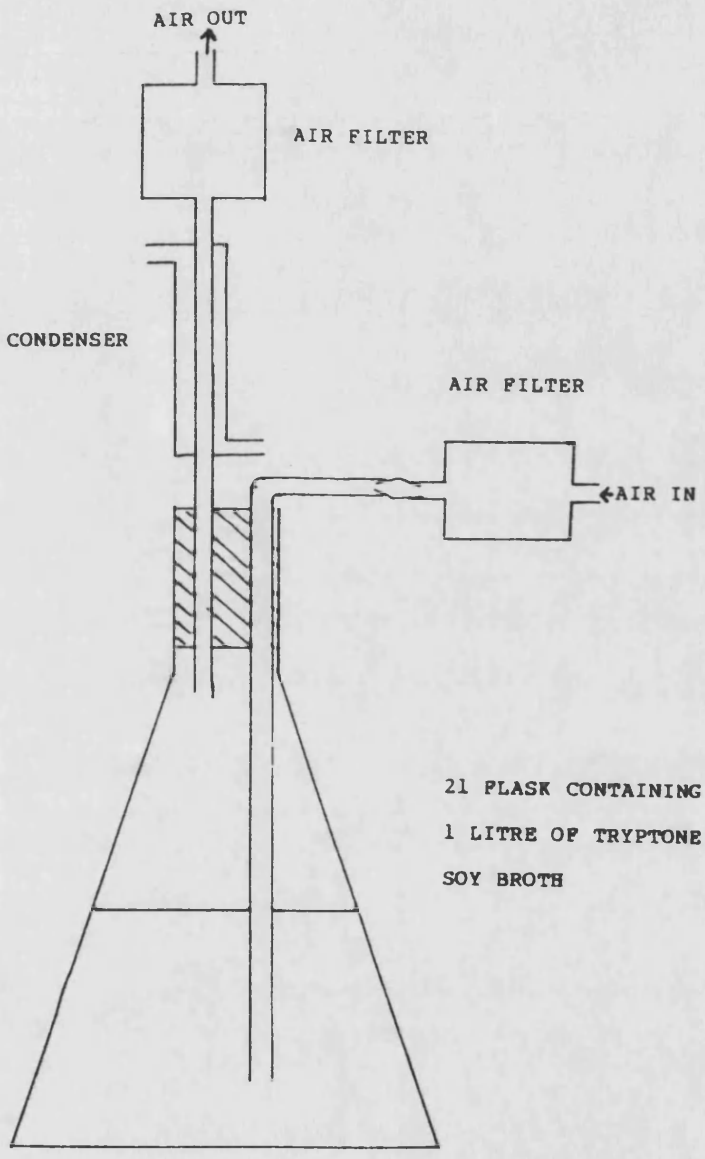
b) Condensers 2-4

Table 4.3 contains the results for the other three condensers. Condenser number 4 was chosen as the most suitable for use with the ALF because it prevented losses of large volumes of liquid and was the shortest condenser. The temperature of the cooling water for the condensers was found to cause a build up of ice on the condensers. Consequently the temperature of the cooling liquid was raised to approximately 1^{o}C.

A larger version of condenser 4 was obtained, air

FIGURE 4.4

The Test Arrangement For Air Filters



it had a 200mm effective length and it was this condenser which was used on the fermenter.

TABLE 4.3

A COMPARISON OF THE EFFECTIVENESS OF THREE DIFFERENT CONDENSERS AT PREVENTING WATER LOSS FROM A 5.5 LITRE AIRLIFT FERMENTER

Condenser	2	3	4
temperature of air entering condenser(^{o}C)	24	24	24
temperature of air leaving condenser(^{o}C)	17.5	9.5	4.5
liquid loss ml day^{-1}	250	185	100
liquid loss rate (ml hr^{-1})	10.4	7.7	4.2
lagged	no	yes	yes

note - lagging consisted of a 3cm thick layer of non absorbent cotton wool wrapped in metal foil.

4.7.3 Air Filter Tests

Whatman gamma 12 cartridge air filters were used to sterilise inlet and outlet air. The suitability of the filters and tube connectors to carry out this task was tested by using a 2 litre flask containing one litre of tryptone soy broth (fig 4.4). Air was blown into the flask using the air filters on the gas inlet and exit lines. No contamination was detected after 2 weeks incubation at 27^{o}C with continuous gas sparging.

4.7.4 Non Return Valve

A stainless steel non return valve was used. If a

back pressure of 1/3 lb per square inch greater than the inlet pressure was experienced the valve shut out. The pressure exerted by the fermenter contents was sufficient to prevent flow back of fluid into the inlet filter of the ALF if the air supply failed.

4.7.5 Air Control

Air was passed through a pressure regulator and the pressure reduced to 10 psig then passed through a gap meter which gave control of air flow between 0 and 12 l min^{-1}.

4.7.6 Temperature Readings

A mercury in glass thermometer was used for temperature measurement.

4.7.7 pH readings

An Ingold pH probe was used in conjunction with a Pye Unicam pH controller.

4.7.8 Oxygen Readings

An EIL galvanic oxygen probe was used in conjunction with an EIL oxygen meter. Initial dissolved oxygen concentration was set at 80% on the meter.

4.7.9 Sampler

A glass 1cm id tube was inserted into one of the bottom ports of the fermenter through a PTFE septum.

This was connected to a modified 1 oz universal sample bottle top using a short piece of silicone rubber tubing (fig 4.5). Normally the tube was sealad using a clamp. the sampling procedure was as follows:-

i Open clamp.

ii Draw the liquid into the sampling bottle using a syringe and fill the bottle to remove medium from the tubing.

iii Replace with a new sterile bottle.

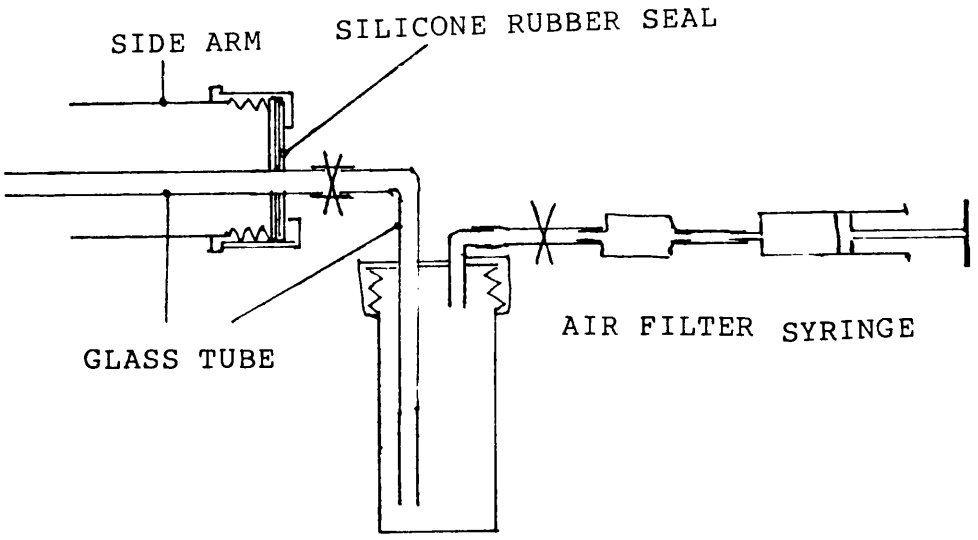
iv Take a sample of the required volume.

v Replace the sample bottle with a new bottle.

vi Blow medium out of the sample tube using a syringe and shut the clamp.

Figure 4.5

The Sampling Arrangement for the ALF.



4.7.10 Filling With Medium and Inoculation

Sterile medium in a large (5 litre) aspirator was heated to 27^{o}C in a water bath, inoculum was added and the medium and inoculum forced into the fermenter through a stericonnector using air pressure. The aspirator was then placed on a shelf at a higher level than the final medium height in the fermenter and allowed to syphon into the fermenter. Air was slowly sparged into the fermenter throughout this process to prevent the nonreturn valve leaking back into the air filter because the initial liquid head in the fermenter was not sufficient to close the valve.

4.7.11 Chemical Sterilisation of The Airlift Fermenter

Seventy percent methanol and alcide were tested as means of sterilising the completed fermenter by spraying with the compounds and then filling with sterile nutrient broth. Neither Alcide nor 70 % methanol were successful as sterilants. A method of steam sterilisation of the fermenter was devised.

4.7.12 Steam Sterilisation

The following items were pre sterilised in autoclave bags - a spare port and seal for one of the outer top ports, a spare port and seal for one side arm, an inlet air filter with non return valve and a condenser outlet filter and port linked as one unit.

Steam was provided from a steam line which was

left open for five minutes to clear out condensation and heat up the line. The open end of the steam line was then inserted into the ALF through a silicon rubber seal in the top plate. A reducing valve was used to control the steam inlet pressure. An initial low inlet pressure (5 psig) was used to warm the fermenter and gradually raised to 30 psig over 30 minutes. All parts of the fermenter were originally loosely fastened but tightened after 1 hour of steaming. Steam egress then occured through the sparger tube and silicon rubber tube which was inserted in one of the side arms. The escape of steam from the sparger was then reduced to a trickle by the use of a clamp on the silicon rubber tube attached to the bottom of the sparger tube. This enabled the use of the side arm tube as a condensate and steam exit.

After two hours the steam pressure was dropped to 5 psig and the sterile bottom filter attached. The steam was turned off and a blank attached to the side arm after removing the exit tube and then the steam inlet line removed and replaced with an intact seal and fittings. When the fermenter had cooled the condenser was placed in the central fitting of the top plate.

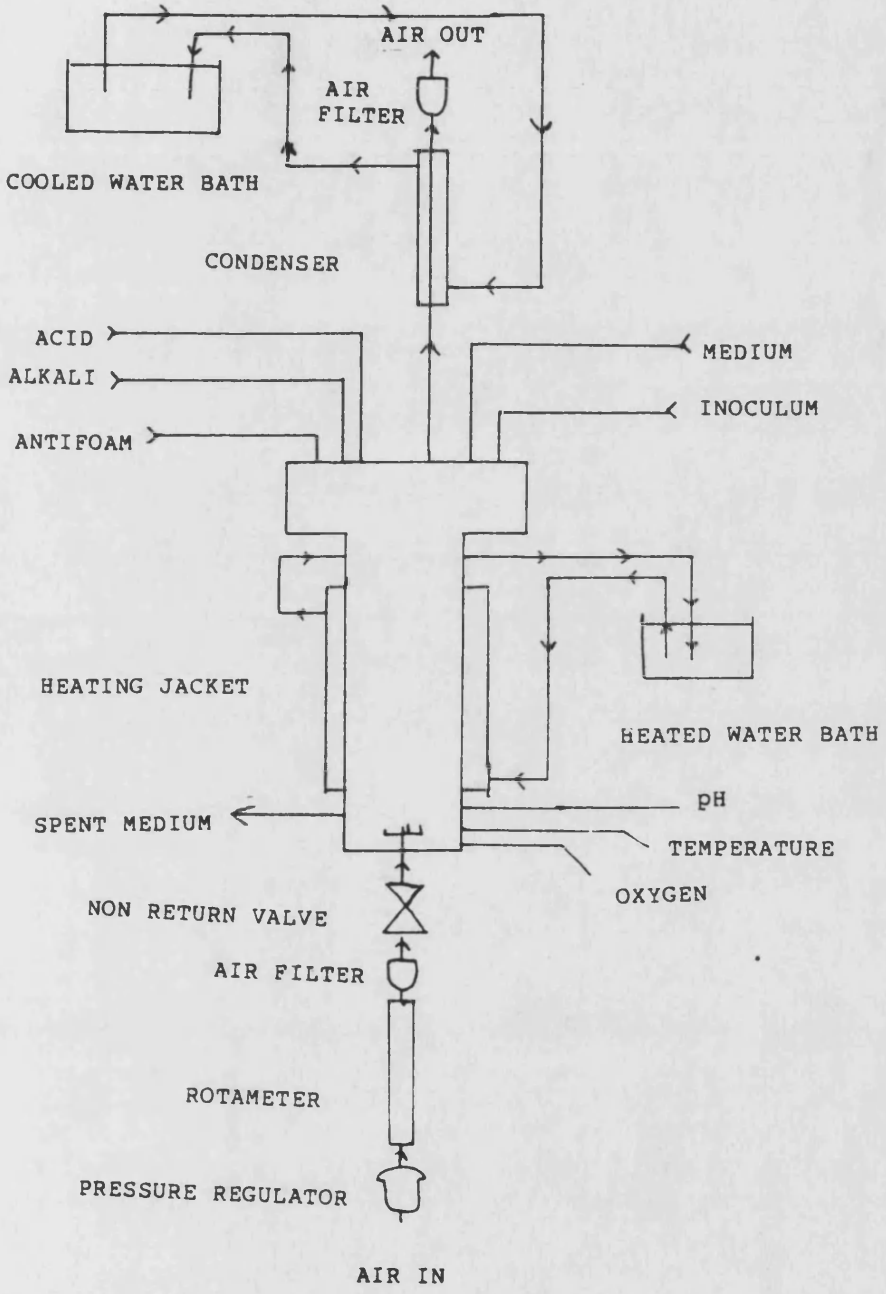
4.7.13 Sterilisation of Accessories

4.7.13.1 pH probe and oxygen probe

These probes were autoclaved with their tips submerged in distilled water.

FIGURE 4.6

A Schematic Diagram of the ALF



4.7.13.2 Samples and aspirator tops

The samplers and aspirator tops were sterilised in autoclave bags.

4.7.13.3 Medium

Mineral salts medium containing caesine hydrolysate was sterilised in the autoclave in a 5 l aspirator. The glucose was autoclaved in a 1l flask and added to the mineral salts medium after sterilisation.

4.7.13.4 Acid, Alkali and Antifoam

All were heat sterilised at 15 psig-121 ^{o}C for 20 minutes. Once the fermenter was connected up to the condenser and water control systems the probes and sampler were placed in the side arms whilst air was being sparged into the fermenter.

Top pressure was then used to add the medium etc.

The final fermenter layout is shown as a schematic diagram in figure 4.6

4.7.14 Liquid Flow in the ALF

Several simple experiments were carried out to determine whether the design used in the construction of the ALF would be suitable for the growth of fungal pellets in suspension. All these experiments were carried out using a liquid volume of 5.5 l

4.7.14.1 Dye

One millimetre aliquots of dye (black chart recorder ink) were ejected from 1mm i.d. silicon rubber tube. Samples injected at the top of the annulus travelled as fairly small markers until they reached the bottom of the draught tube where they mixed rapidly in the bottom space. This indicated that the downward flowing liquid in the annulus was of a laminar type where as the bottom section of the liquid column was well mixed.

Dye added to the bottom of the draught tube flowed upwards but was more dispersed than material travelling down the outside of the draught tube. Once the dye reached the top of the fermenter it mixed quickly. Dye added to the disengagement area and the sparger area was quickly dispersed in those areas, thus indicating that the flow up the centre of the draught tube was better mixed than the flow down the outside and that the disengagement volume was well mixed.

The time taken for the dye to get from the top of the draught tube to the bottom was in the region of 1 to 3 seconds. This gives a liquid velocity in the region of 0.5 to 0.17 m s^{-1}.

4.7.14.2 Killed Fungal Pellets

Killed pellets from shake flasks were circulated at varying air flow rates. Air flow rates of greater than 3 or 4 litres min^{-1} kept most of the pellets

suspended but some pellets lodged on the draught tube side supports. Air bubbles and pellets were very difficult to tell apart when the fermenter was operating and no circulation times for suspended pellets could be reliably estimated.

4.7.14.3 Plastic Square

A 1 cm square of plastic of almost neutral buoyancy was placed in the fermenter. Although the square was black it was still very difficult to distinguish from air bubbles. Small bubbles attached to the surface of the square, making it buoyant and rendering data obtained from the square useless.

4.7.14.4 Polystyrene Cube

A cube of approximately neutral buoyancy was constructed and found to suffer from none of the disadvantages of the killed pellets or the plastic square. This cube was found to circulate at most air flow rates. The effects of air flow rates and liquid volume in the fermenter were now measured using this pellet.

a) Optimum Liquid Height and Air Flow Rates

A one cubic centimetre cube of approximately neutral buoyancy was constructed using expanded polystyrene and copper wire. The time taken for 10 circuits of the fermenter by the cube was measured at

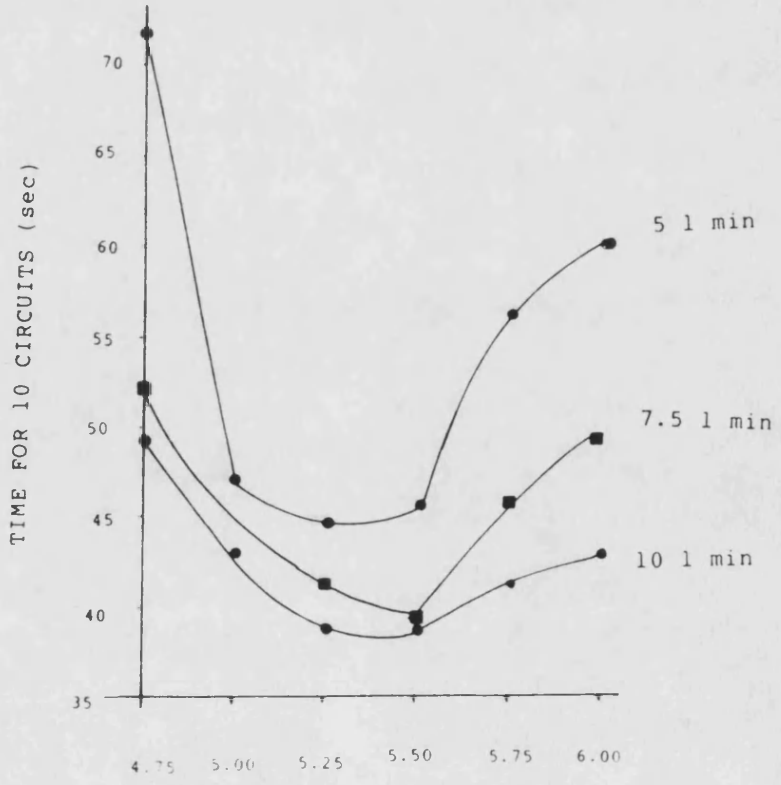
different water levels and air flow rates. A draught tube with 5 cm long legs was used.

As can be seen from fig 4.7 there was a definite relationship between air flow rate, liquid volume (and hence height) and circulation rate.

At each air flow rate the optimum liquid volume for maximum circulation rate was approximately 5.5 l. A low liquid level resulted in the particle having a long residence time in the zone at the bottom of the draught tube. The increase of air flow rate at low liquid volumes increased the circulation rate by increasing the gas holdup in the fermenter. Large liquid volumes resulted in the retention of the particle in the medium above the top of the draught tube. This volume was well mixed and the volume was large enough to prevent very rapid turnover of the contents of the disengagement volume.

Figure 4.7

The Relationship Between Air Flow Rate, Liquid Volume and Circulation Rate in the ALF



CHAPTER 5

FERMENTER EXPERIMENTS

5.1 General Introduction

There is a considerable literature on the culture of filamentous organisms in fermenters (eg 5,6,16,20,21,25,30,43,97,106,124,125). Much of the literature is concerned with the effects of heavy concentrations of filamentous biomass on mass transfer within the fermentation medium. Mycelia, small pellets and dense cultures of single celled organisms tend to produce non Newtonian liquid flow in fermentation liquors (5,26,35,59,89,120,136). Non Newtonian liquids require greater power input per unit volume to achieve suitable mixing for fermentation than Newtonian liquids in the same vessel because of the greater viscosity of the medium. As a result adequate mass transfer and hence control of the fermentation are difficult to achieve (26,87,120). The high stirrer speeds necessary to allow adequate mass transfer to occur may also cause damage to the fungal mycelia because of the high shear rates at the tip of the impeller (99,100,120,121,128).

Although the STR type of fermenter is the one most commonly used to grow viscous mycelial growth forms there are reports of ALF's being used for the same purpose (8,9,64,103). However, most authors agree that viscous growth media are not suitable for use in airlift type vessels. This is because

a) Small bubbles are held up in the viscous medium. The holding time in the disengagement volume is not long enough to allow disengagement of many of these small bubbles (36). This reduces the efficiency of mixing by reducing the density difference between the medium in the riser and downcomer (10).

b) Larger bubbles rise rapidly but have little effect on the medium directly to either side of them. The air flow can be considered as slug rather than bubbly flow (10,52).

Work with Absidia corymbifera showed that medium containing mycelia exhibited pseudoplastic behaviour. When pellets of 0.5 to 1.5 mm in diameter were used the medium exhibited Newtonian behaviour until more than 28 g l^{-1} of biomass was present, then the rheology changed to pseudoplastic (59). As it is possible to grow fungi such as Penicillium chrysogenum for over 100 hours and the size of the pellets not exceed 1mm diameter (136) it was hoped to be able to grow C.bainieri in a similar form. If the pellets could also be induced to fragment the fungus would be ideal for continuous culture in both the fermenters used. The removal of small pellets in continuous culture would be easier than trying to remove filaments as they tend to snag on obstructions and block weirs. Pellets have a tendency to sediment out but this problem can be overcome by the use of correct agitation and medium removal methods.

CHAPTER 5

The aim of the fermenter experiments was to compare fungal growth in the two different fermenters. Hopefully, a comparison between pelleted and filamentous growth in both fermenters could also be carried out. Filamentous growth would also have provided opportunities for continuous culture to be used for detailed investigation of the effects of culture conditions on growth.

5.2 Materials and Methods

The materials and methods which were not described in chapters 2 to 4 are described here. They are materials and methods used with the STF.

5.2.1 Fermenters

A 12 l nominal volume LH 2000 series STR and a 5.5l working volume airlift fermenter were used for this work.

5.2.2 Environmental Control

The fermenter was equipped with automatic pH, temperature, dissolved oxygen, stirrer speed and antifoam controls. These were used with a varying degree of success.

5.2.2.1 Antifoam Control

Several attempts were made to use the antifoam control system but fungal overgrowth on the probe led to continuous pumping. As a consequence the antifoam controller was disconnected and 100 ppm of antifoam B was added to the medium prior to sterilisation. Further doses of antifoam were added through the septum of one of the top ports using a sterile hypodermic syringe and needle. The additional doses were only necessary when the stirrer was controlled at 1000 rpm.

5.2.2.2 Dissolved Oxygen

The dissolved oxygen tension could be controlled by two different methods, continuous air flow with variable stirrer speed and intermittent air flow with constant stirrer speed. It was necessary to maintain a constant air flow rate to keep the sparger clear of fungus. A constant stirrer speed was also needed so that comparisons could be made between different stirrer speeds. The control unit was set to intermittent sparging and the sensitivity of the instrument set to a level at which the air supply would be continuous.

5.2.2.3 pH control

pH control on the STR ran into two problems- The correct diameter tubing for use in the peristaltic pumps attached to the fermenter was difficult to obtain. The correct pressure could not be maintained on the tubing and acid and base leaked into fermenter before

air sparging was started. When air sparging was initiated the air blew back into the reservoirs.
Fungal growth on the probe made the readings from the probe unrepresentative of the fermenter medium as a whole. Medium samples were therefore taken and their pH measured using a portable pH meter.

5.2.2.4 Temperature

The automatic temperature control system was used to control the fermenter without any alterations. A mercury in glass thermometer was used to check the thermistor before starting the fermentation. Temperature control was adequate even when one of the two heating fingers burnt out.

5.2.3 Sterilisation

The fermenter was designed for in situ sterilisation using its heating elements. Appropriate volumes of mineral salts solution were placed in the fermenter and the fermenter sterilised according to the manufacturers instructions. A temperature of 121^{o}C was used for half an hour. The air exit filter was lagged to help prevent heat loss. Liquid loss through the steam trap was measured and replaced with sterile distilled water. Other nutrients were sterilised seperately.

5.2.4 Sampling

Samples were taken via a valve in the bottom of the fermenter. Originally it was hoped to use a sampler like the one used on the ALF, using one of the fermenters sample tubes. Growth of fungus in the sample tubes prevented this sampling method being used.

A valve at the bottom of the fementer which was intended for drainage of the contents after sterilisation was therefore chosen for sampling purposes. The valve was washed with 70% alcohol and flamed before and after sampling.

5.2.5 Fermentation Conditions

5.2.5.1 The ALF

a) Spargers - A sintered sparger was used for runs 1,2 and 4. A single orifice sparger was used for run 3 and the four orifice spider sparger was used for the other runs.

b) Draught tubes - The draught tube support ring was used for the first five runs. The height between the bottom of the draught tube and the fermenter bottom was 10 cm in runs 1 to 4 and 6 and 7. In the other runs the gap was 5 cm.

c) Air Flow rate - A constant flow of 5 l min^{-1} of air

was maintained throughout the fermentations, except a transient increase after 24 hours, to resuspend any material settled at the bottom of the fermenter.

d) Antifoam - one hundred ppm of antifoam B were added to the medium prior to the first four runs. The remaining runs were performed in the absence of antifoam.

e) Inoculum - Ten day old spores were used for the inoculum. Run 1 was inoculated with $5x10^4$ spores ml^{-1}. Runs 2 to 4 were inoculated with $3x10^4$ spores ml^{-1}. The other runs were inoculated with spores taken from a single petri dish. Runs 5 to 7 were not pregerminated, runs 8 and 9 were pregerminated for 4 hours and the remaining inocula were pregerminated for 24 hours. All pregerminations were carried out in shake flasks at 27^oC, 250 rpm and using 50 ml of malt extract broth in a 250ml Erlenmeyer flask.

5.2.5.2 The STF

a) Air sparging - intermittent air sparging was used for the first two runs. All subsequent runs used constant air flow rates. The air flow rates were 7 l min^{-1} for runs 1 - 5, 10 l min^{-1} for runs 6 - 8 and 2.5 l min^{-1} for the rest of the runs.

b) Liquid volume - A liquid volume of 7l was used in runs 1-3, 8l in run 4, 9l in run 5, 8.5 in runs 6-7 and 9.5i in the remaining runs.

c) Stirrer speed - runs 1 - 3 were stirred at 700 rpm, runs 4 - 9 were stirred at 1000 rpm and the rest of the runs were stirred at 500 rpm.

d) Antifoam - antifoam was used in runs 4 - 9.

e) Inocula - Five flasks of two day primary cultures grown as for the shake flask experiments (chapt 2) were used to inoculate runs 1 - 5. Flasks containing pulpy mycelial growth were chosen preferentially. Run 6 was inoculated using $1.5x10^5$spores ml^{-1}. Runs 7 - 10 were inoculated using $3x10^3$spores ml^{-1}. $9x10^6$spores ml^{-1}were used for run 11 and $5x10^4$spores ml^{-1}were used for run 12. Pregerminated spores as described in the previous section were used for the rest of the runs. The inoculum for run 13 was pregerminated for 4 hours. The other inocula were pregerminated for 24 hours.

5.3 Results and Discussion

5.3.1 Growth Data

The growth data is presented in the following section with tables figures and a brief description of the results for each run. Total glucose depletion was not achieved and in the runs which continued for any time

attached growth was always a problem. STR experiments marked * X where X is a number were run as paired experiments with the numbered ALF run.

5.3.1.1 STF (LH Fermenter Results)

Unless otherwise stated the predominant growth form in the fermenter was attached with a small number of pellets in suspension

Run 1

Intermittent air sparging was used. The sparger and sample tubes quickly became filled with fungus. Contamination was apparent after 3 days.

Run 2

Intermittent air sparging was used. The sparger and sample tubes quickly became filled with fungus. Contamination was apparent after 4 days.

Run 3

The liquid in the fermenter was totally clear after 3 days with NO pelleted growth. Most of the attached growth grew on the baffles.

Run 4

A very small amount of norcodeine was detected after 6 days Figs (5.1-5.3). Most of the growth was attached by the end of the fermentation.

Run 5

A broken seal caused leakage and contamination was detected after 3 days.

Run 6

Growth was originally as dispersed mycelia which rapidly rose to the top of the medium and formed a lid of fungus. After 2 days 3l of liquid had been lost due to foaming leaving a layer of fungus several centimetres above the level of the medium.

Run 7 (* ALF 2)

Small pellets formed but within two days these had attached themselves to the fermenter. There was a mycelial ring around the bottom of the fermenter. After four days four liters of medium had been lost as a result of heavy foaming. (figs 5.4-5.6).

Run 8

Foaming was very bad and 2.5 liters of medium were lost in 2 days the run was terminated.

Run 9 (* ALF 4)

A final concentration of 500 ppm of antifoam was added to the fermenter over 3 days. This did not prevent foaming for more than 1 or 2 hours after the addition of each new batch of antifoam. There was very little visible growth in this fermenter and less than 4.5 l of medium remained after 5 days fermentation (figs 5.7-5.9).

Run 10 (*ALF 5)

Lowering the stirrer speed prevented foaming. Only two pellets were seen, the rest of the growth being attached (figs 5.25-5.28). There was little liquid loss over the 10 day period of the fermentation. Glucose uptake was very slow (figs 5.10-5.12)

Run 11 (*ALF 6)

This run was very similar to run 10 but more small pellets were produced and after 4 days the fermenter was dismantled and photographed (figs 5.29-5.32).

Run 12

Growth was predominantly attached and the fermentation was halted after 3 days.

Run 13 (*ALF 9)

Small plentiful pellets were produced (figs 5.13-5.15).

Runs 14 - 15

Same as run 12.

Run 16 (ALF 11)

Pellets were produced in this run but a crust of biomass formed at the surface of the medium (figs 5.16-5.18).

Runs 17- 19

Same as run 12

Runs 20-21

Pellets and attached growth were produced but the growth was mainly attached and the runs were terminated after 4 days.

Run 22

There was heavy growth on the surfaces of the fermenter (figs 5.22-5.24).

5.3.1.2 Airlift Fermenter Results

Run 1

Pelleted growth was produced but the fermenter became contaminated after 2 days. The contaminent reduced the dissolved oxygen tension to zero within a day.

Run 2

A small number of large pellets were produced. They settled readily and had to be repeatedly resuspended.
After 5 days there was a large accretion of fungus around each of the draught tube top supports and the support ring of the draught tube. There was also a mycelial mat on the sparger and a mat of fungus over the dissolved oxygen probe (figs 5.4-5.6).

Run 3

Some half centimetre pellets were produced after 3 days but in general the growth was attached and little growth was detected.

Run 4

Lots of small pellets were produced but the support ring began to trap them. After 4 days the

fermenter leaked because the condenser iced up. This caused an increase in pressure and the medium leaked out of the joint between the bottom and mid section of the fermenter (figs 5.7-5.9).

Run 5

A ring formed around the bottom of the sparger and the draught tube. Eventually liquid flow was cut off completely (figs 5.33-5.34). Glucose uptake was very slow (figs 5.10-5.12).

Run 6

Pelleted growth ocurred (figs5.35,5.36) the fermentation was stopped because the draught tube became congested after liquid loss.

Run 7

The results were identical to run 6.

Run 8

Similar to run 6.

Run 9

Lots of pellets and some large clumps were formed. When material which had settled after the first 24 hours of the fermentation was resuspended it

resuspended as a single large mass. Norcodeine (0.4 mg ml^{-1} was detected after 8 days (figs 5.13-5.15).

Run 10

The fermentation looked promising but had to be stopped after 3 days because of liquid loss.

Run 11

The original form of growth was pelleted but this settled after 48 hours. Settled material was resuspended after 48 hours. This produced a few large pellets which caused blockages by becoming wedged between the draught tube and the fermenter wall (figs 5.16-5.18).

Run 12

Most of the fungus was pelleted with some attached to the bottom of the draught tube. Glucose uptake was rapid (figs 5.19-5.21) and 0.51 mg ml^{-1} of norcodeine was detected after 8 days.

Run 13

Rapid pelleted growth occurred but the fermentation had to be stopped because of liquid loss (figs 5.19-5.21).

FIGURE 5.1

Glucose uptake (mg $ml^{-1}x10$) against time for STR run 4.

FIGURE 5.2

Log of glucose uptake (mg $ml^{-1}x10$) against time for STR run 4.

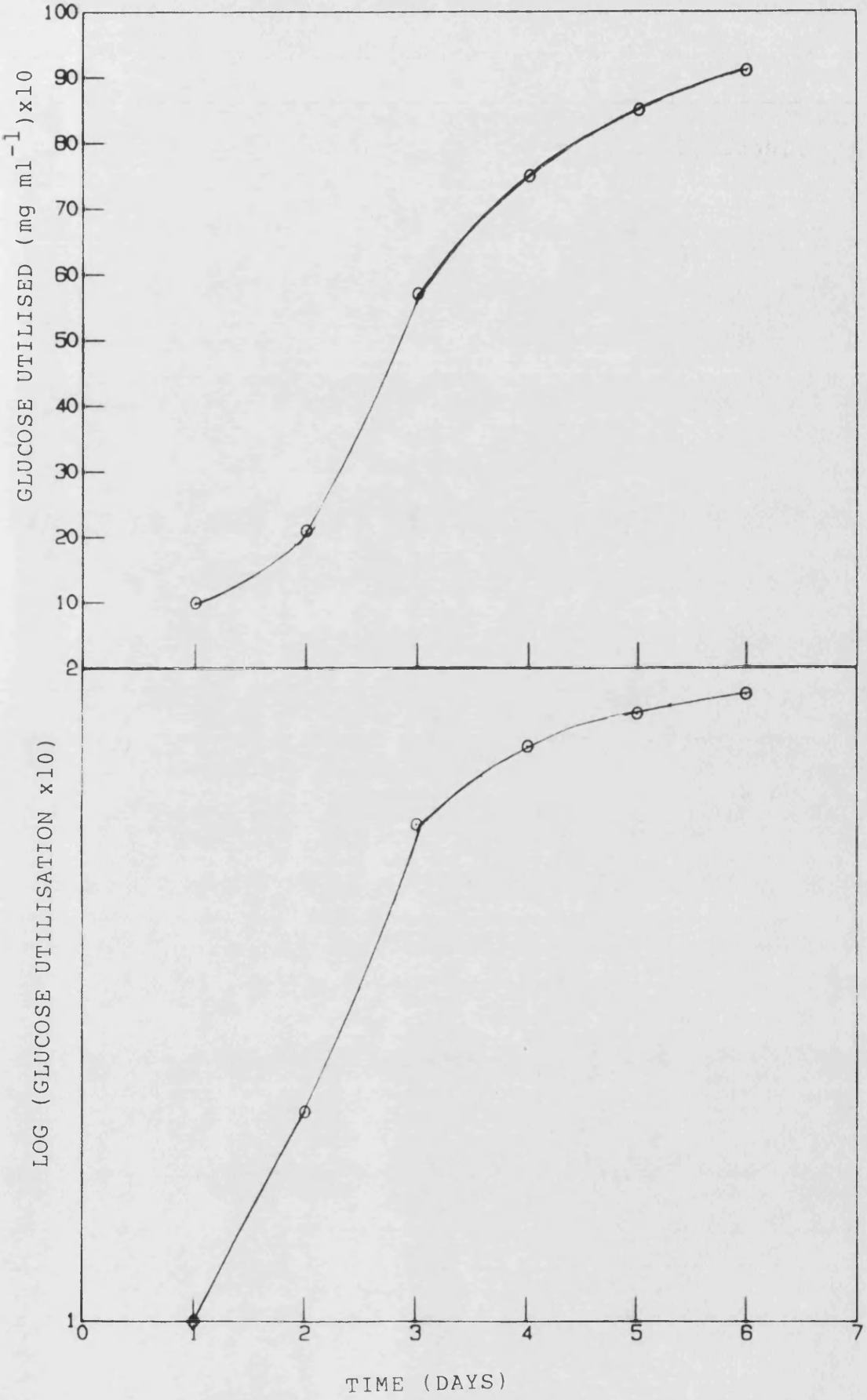
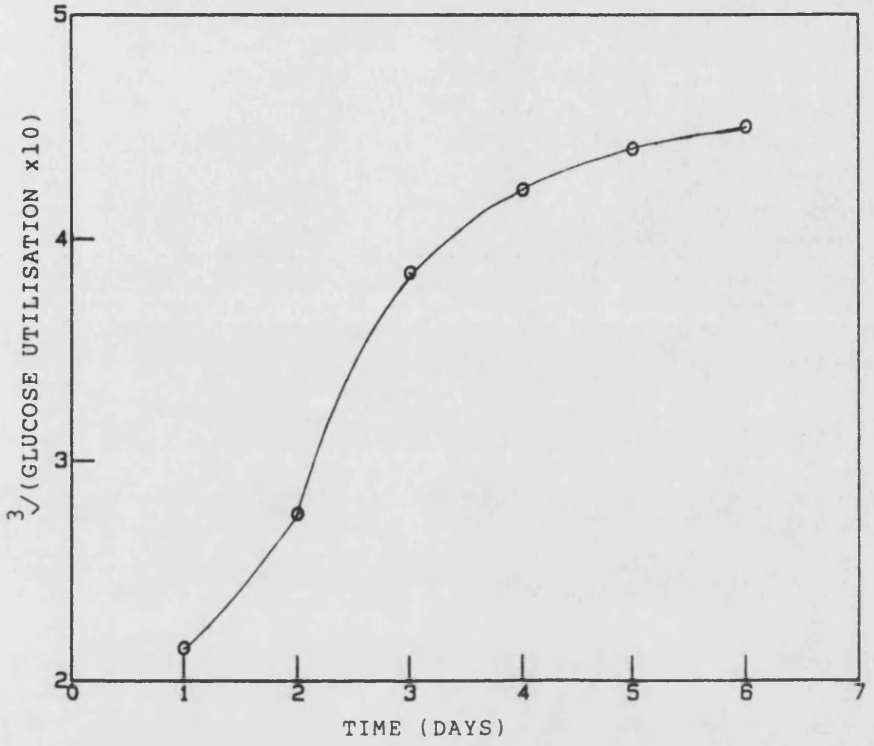

GLUCOSE UTILISED (mg ml^{-1})x10
100
90
80
70
60
50
40
30
20
10
2
LOG (GLUCOSE UTILISATION x10)
2
1
0
1
2
3
4
5
6
7
TIME (DAYS)

FIGURE 5.3

Cube root of glucose uptake (mg ml^{-1}x10) against time for STR run 4.

FIGURE 5.4

Glucose uptake (mg ml^{-1}x10) against time for ALF run 2 (o) and STR run 4 (□).


$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
TIME (DAYS)

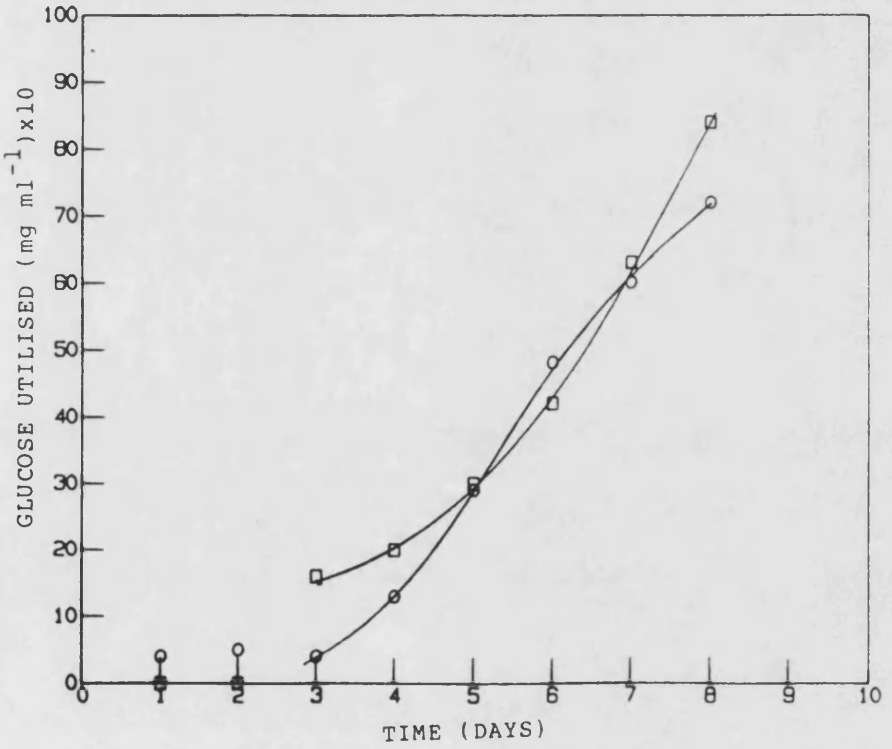

GLUCOSE UTILISED (mg ml^{-1})x10
TIME (DAYS)

FIGURE 5.5

Log of glucose uptake (mg $ml^{-1}x10$) against time for ALF run 2 (○) and STR run 4 (□).

FIGURE 5.6

Cube root of glucose uptake (mg $ml^{-1}x10$) against time for ALF run 2 (○) and STR run 4 (□).

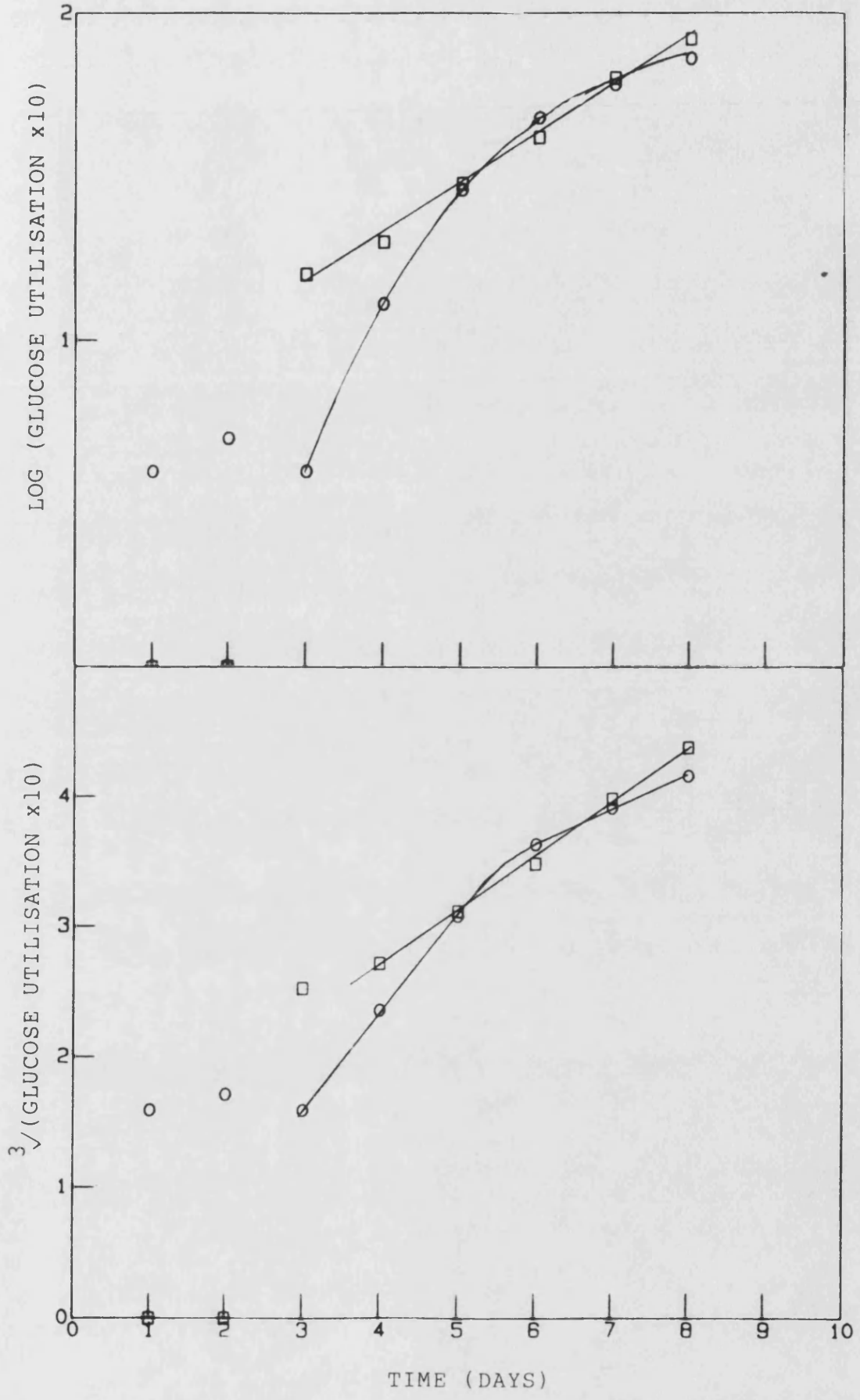

LOG (GLUCOSE UTILISATION x10)
2
1
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
4
3
2
1
0
0
1
2
3
4
5
6
7
8
9
10
TIME (DAYS)

FIGURE 5.7

Glucose uptake (mg ml^{-1}x10) against time for ALF run 4 (o) and STR run 9 (□).

FIGURE 5.8

Log of glucose uptake (mg ml^{-1}x10) against time for ALF run 4 (o) and STR run 9 (□).

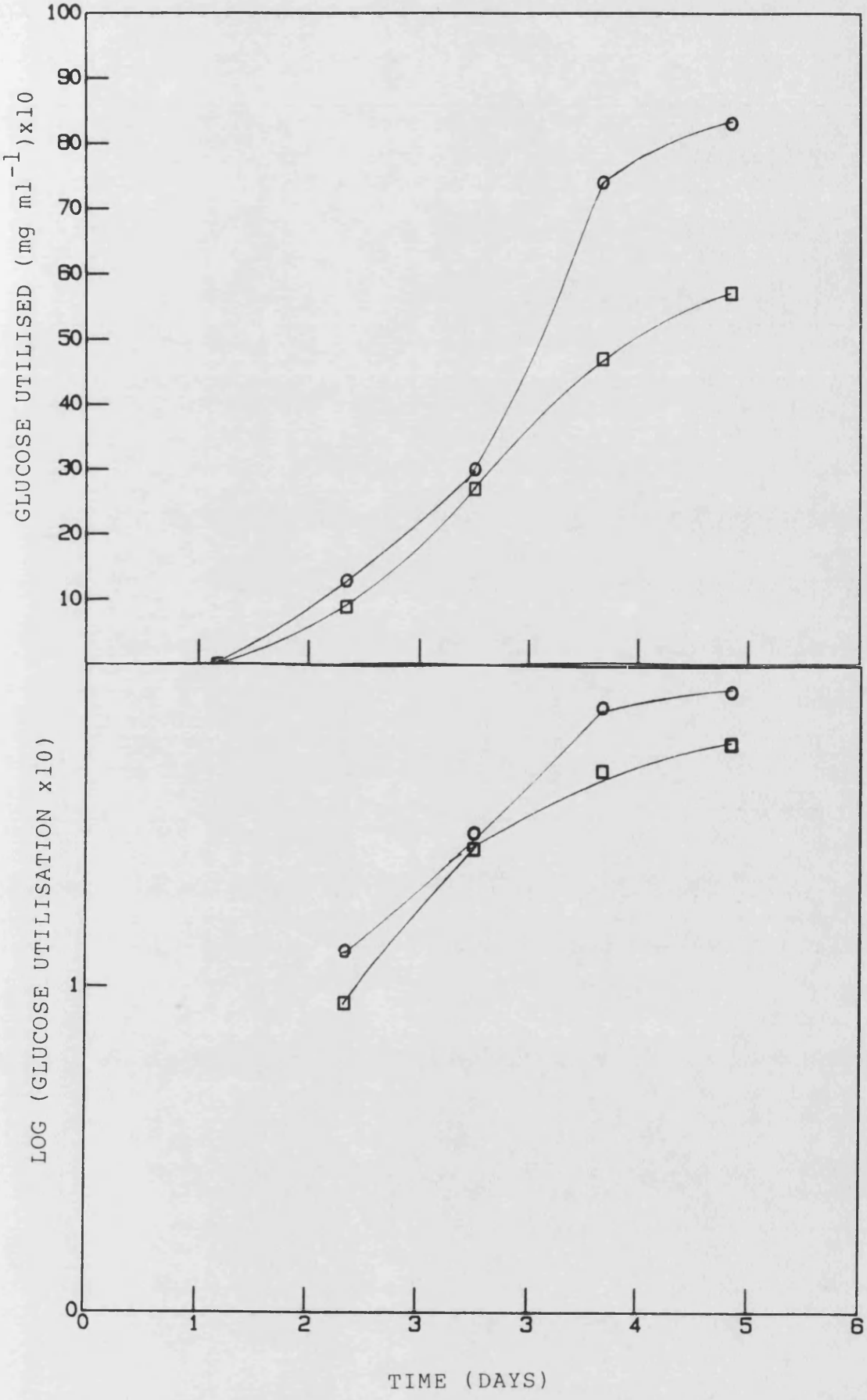
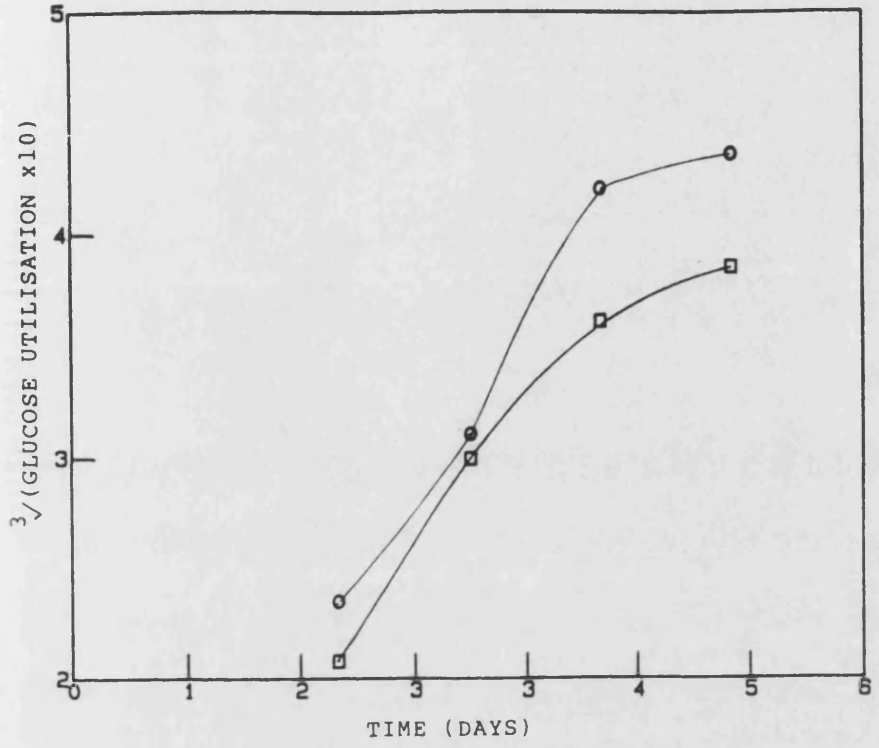

GLUCOSE UTILISED (mg ml^{-1})x10
100
90
80
70
60
50
40
30
20
10
LOG (GLUCOSE UTILISATION x10)
1
0
0
1
2
3
3
4
5
6
TIME (DAYS)

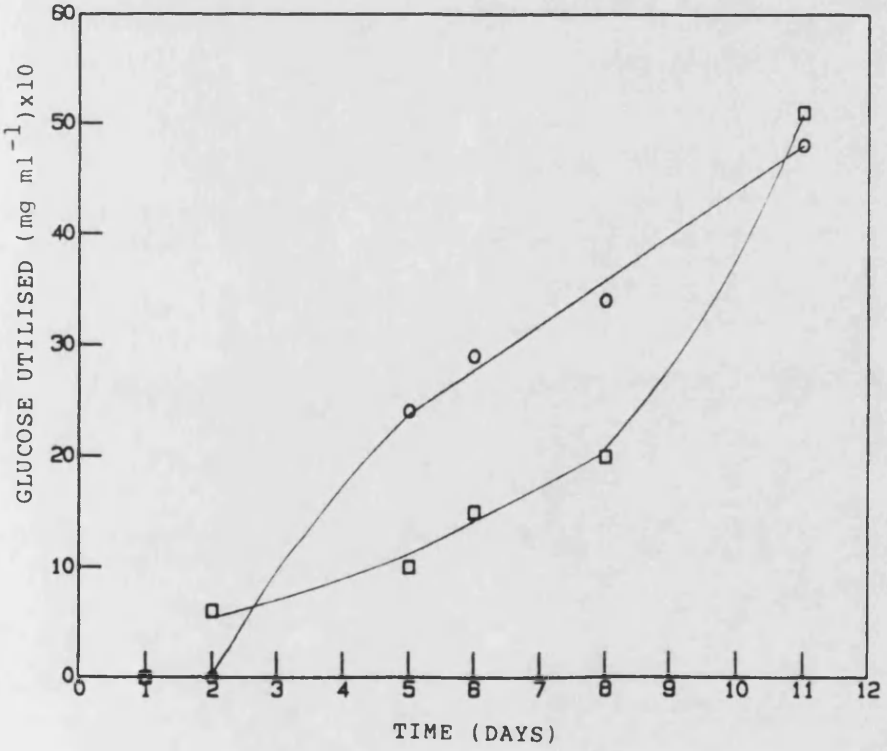
FIGURE 5.9

Cube root of glucose uptake (mg ml^{-1}x10) against time for ALF run 4 (o) and STR run 9 (□).

FIGURE 5.10

Glucose uptake (mg ml^{-1}x10) against time for ALF run 5 (o) and STR run 10 (□).


5
4
3
2
0
1
2
3
3
4
5
6
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
TIME (DAYS)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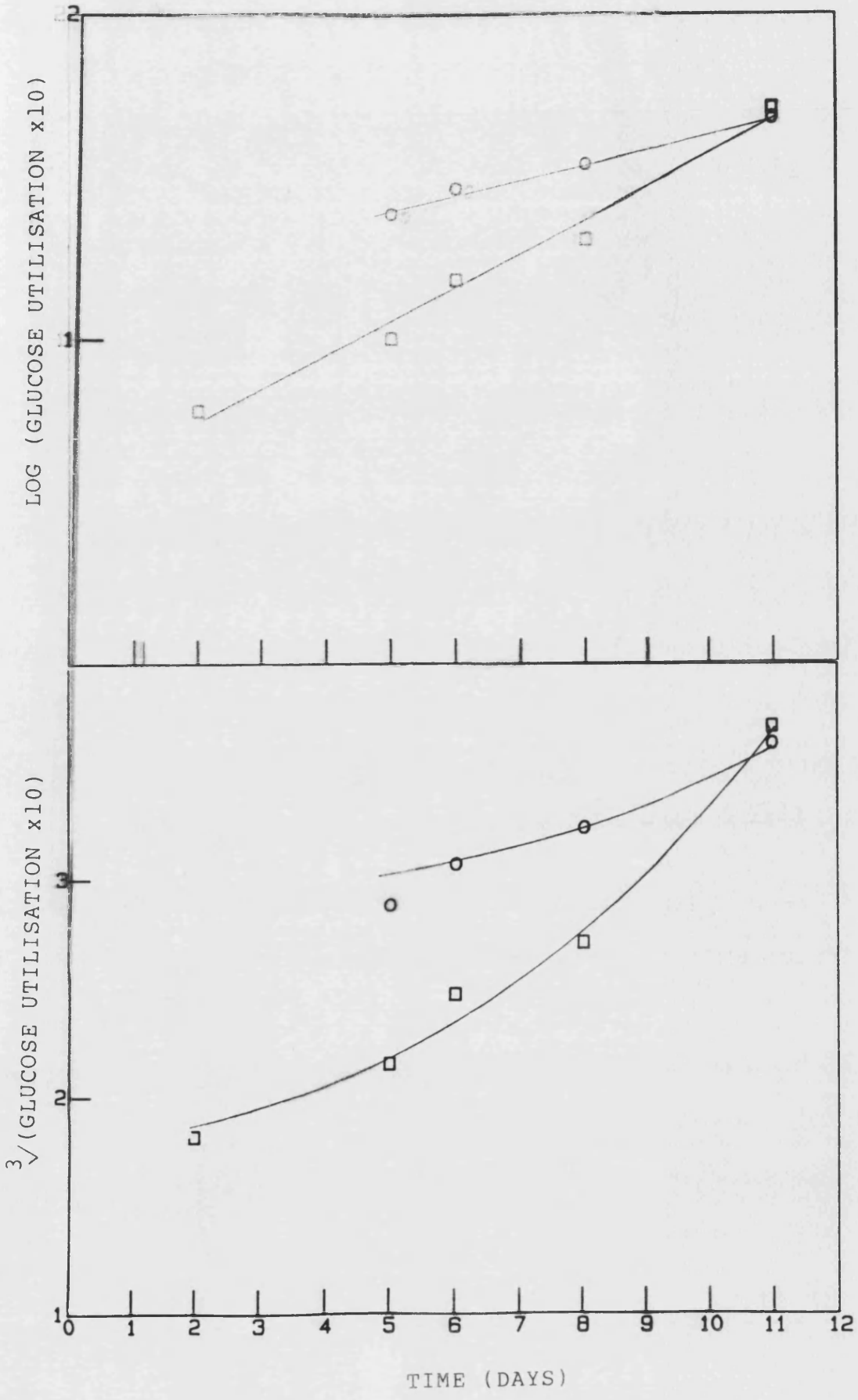
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
11
12
GLUCOSE UTILISED (mg ml^{-1})x10
TIME (DAYS)

FIGURE 5.11

Log of glucose uptake (mg ml^{-1}x10) against time for ALF run 5 (o) and STR run 10 (□).

FIGURE 5.12

Cube root of glucose uptake (mg ml^{-1}x10) against time for ALF run 5 (o) and STR run 10 (□).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1
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
TIME (DAYS)

FIGURE 5.13

Glucose uptake (mg ml^{-1}x10) against time for ALF run 9 (O) and STR run 13 (□).

FIGURE 5.14

Log of glucose uptake (mg ml^{-1}x10) against time for ALF run 9 (O) and STR run 13 (□).

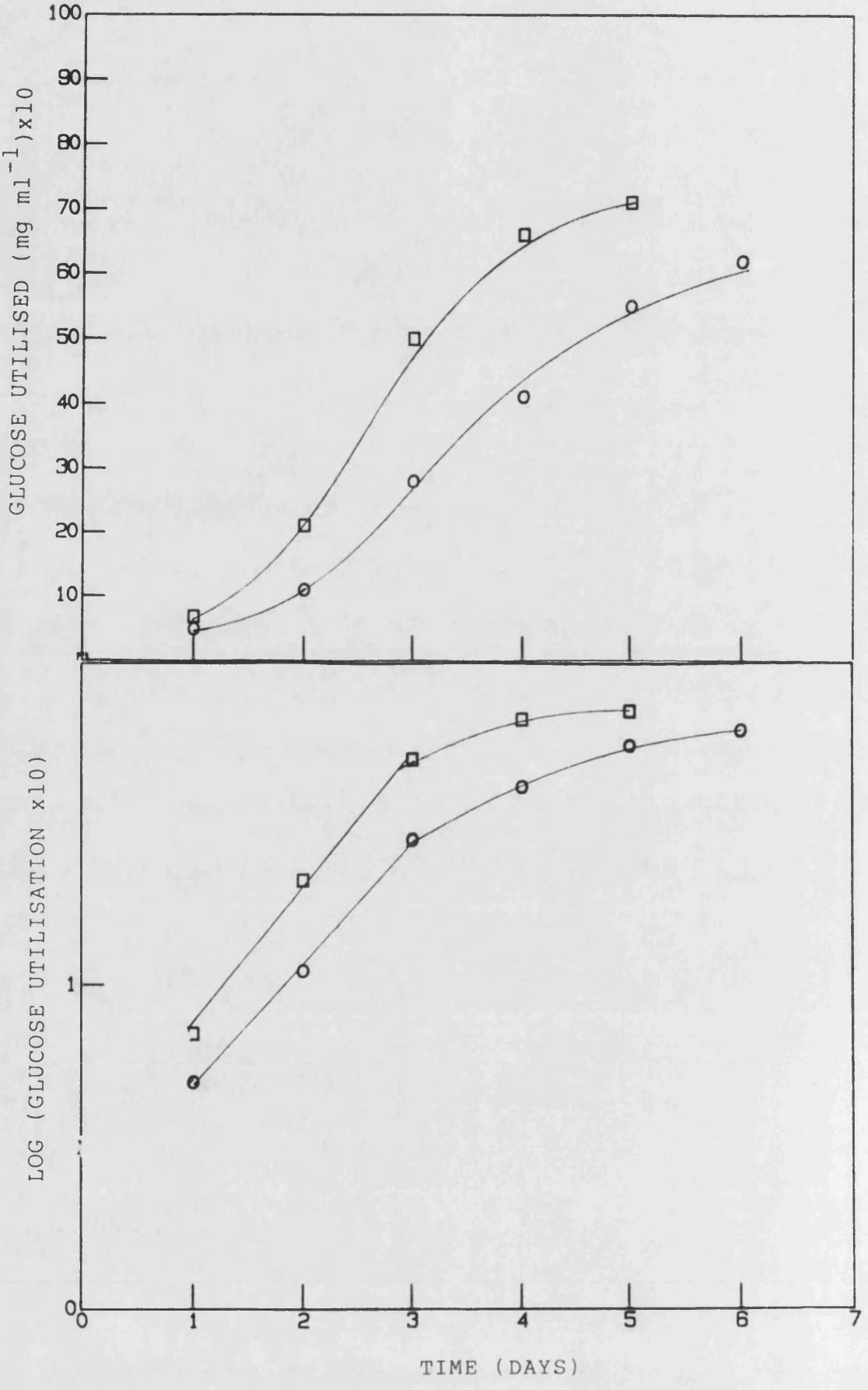
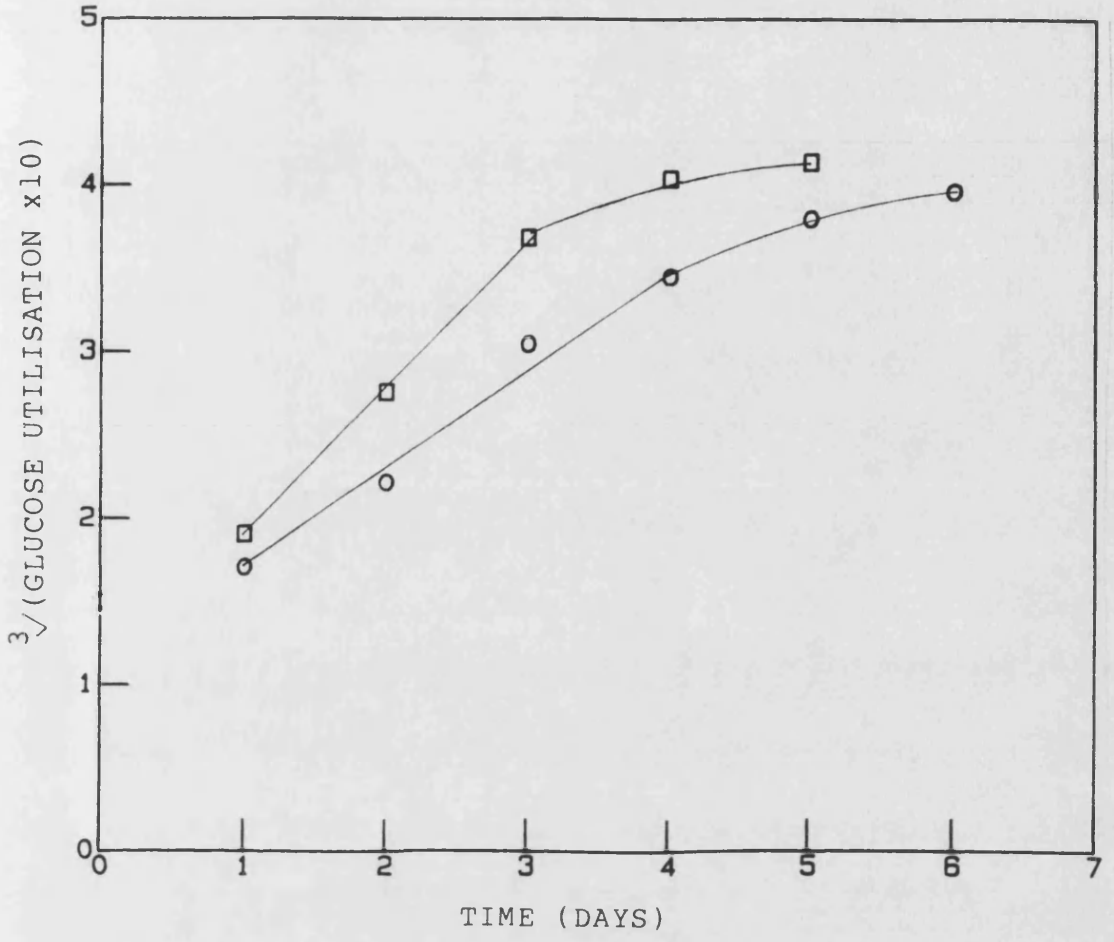

GLUCOSE UTILISED (mg ml^{-1})x10
100
90
80
70
60
50
40
30
20
10
LOG (GLUCOSE UTILISATION x10)
1
0
0
1
2
3
4
5
6
7
TIME (DAYS)

FIGURE 5.15

Cube root of glucose uptake (mg ml^{-1}x10) against time for ALF run 9 (o) and STR run 13 (□).

FIGURE 5.16

Glucose uptake (mg ml^{-1}x10) against time for ALF run 11 (o) and STR run 16 (□).


$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
TIME (DAYS)

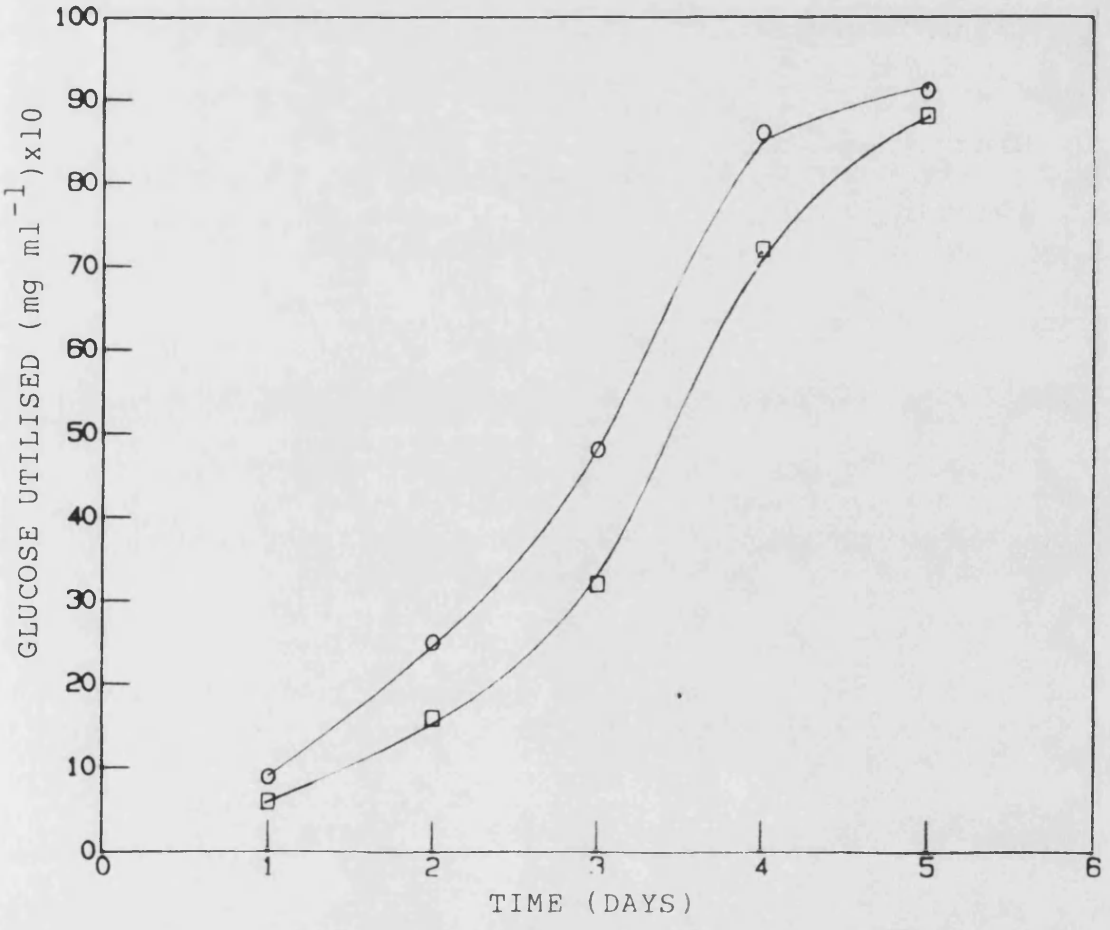

GLUCOSE UTILISED (mg ml^{-1})x10
TIME (DAYS)

FIGURE 5.17

Log of glucose uptake (mg $ml^{-1}x10$) against time for ALF run 11 (O) and STR run 16 (□).

FIGURE 5.18

Cube root of glucose uptake (mg $ml^{-1}x10$) against time for ALF run 11 (O) and STR run 16 (□).

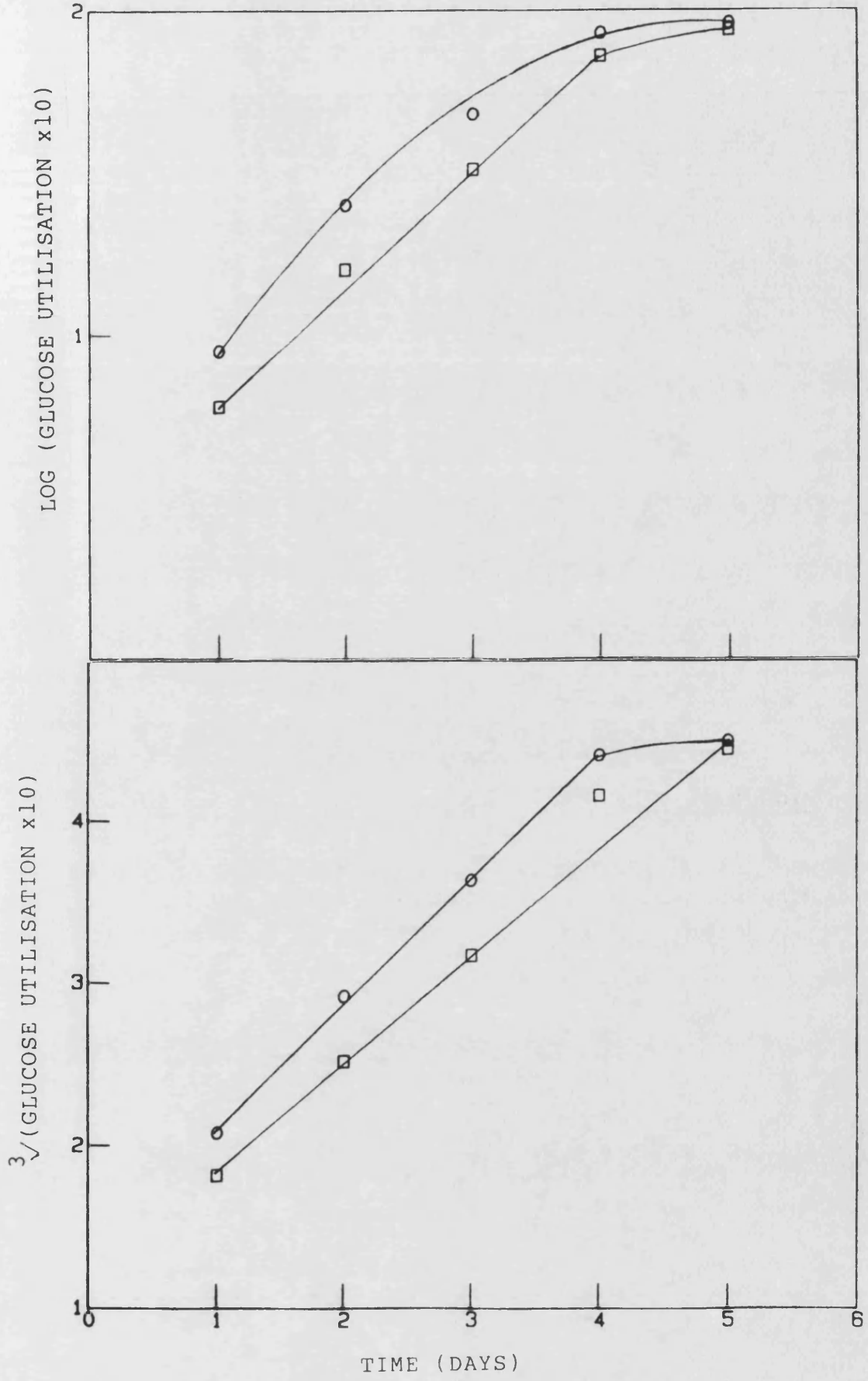

LOG (GLUCOSE UTILISATION x10)
2
1
$\sqrt[3]{}$(GLUCOSE UTILISATION x10)
4
3
2
1
0
1
2
3
4
5
6
TIME (DAYS)

FIGURE 5.19

Glucose uptake (mg ml^{-1}x10) against time for ALF run 12 (O) and ALF run 13 (●).

FIGURE 5.20

Log of glucose uptake (mg ml^{-1}x10) against time for ALF run 12 (O) and ALF run 13 (●).

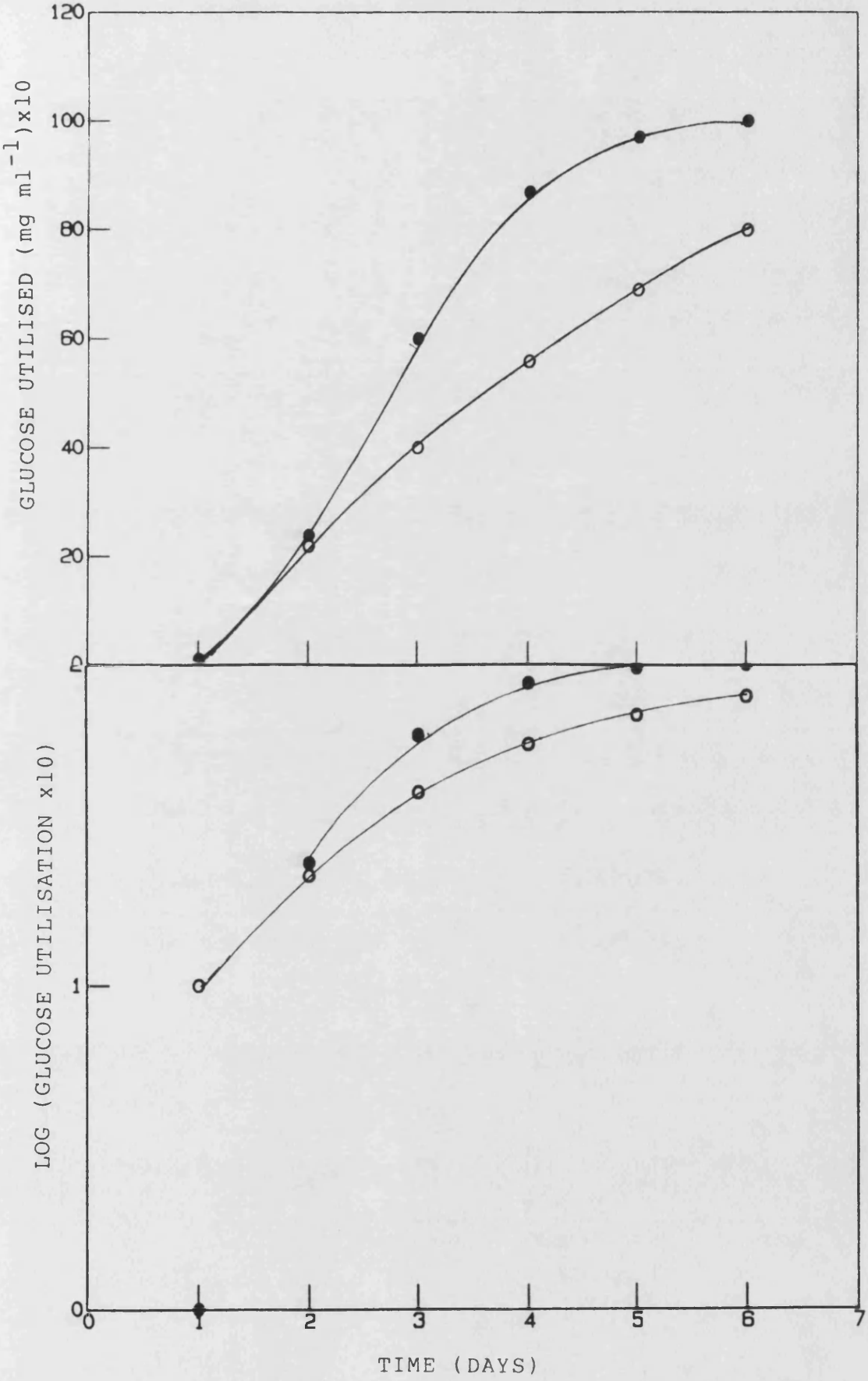
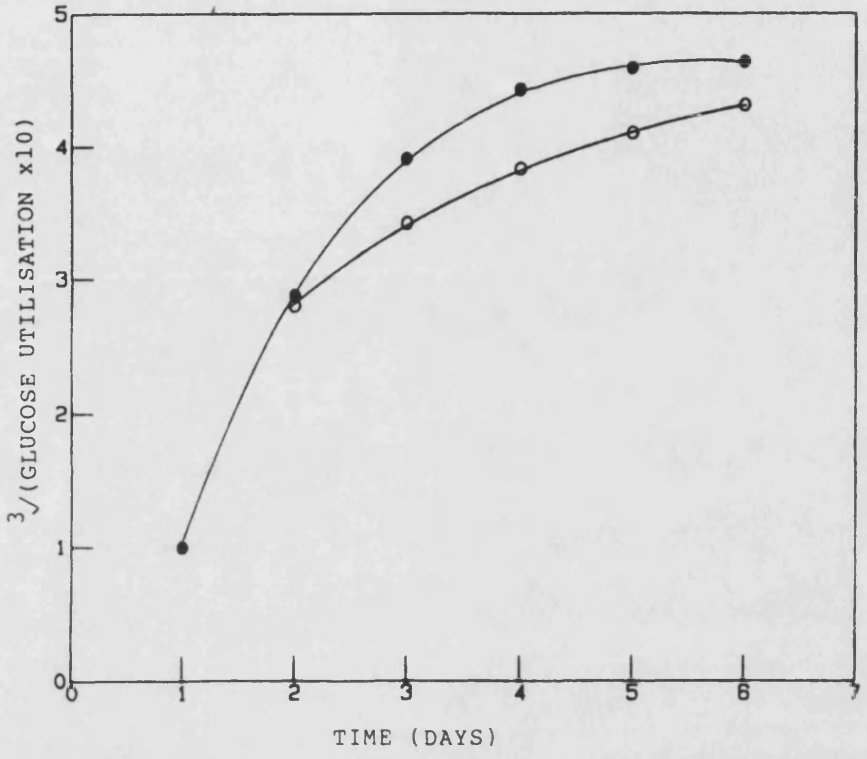

GLUCOSE UTILISED (mg ml^{-1})x10
120
100
80
60
40
20
0
LOG (GLUCOSE UTILISATION x10)
2
1
0
0
1
2
3
4
5
6
7
TIME (DAYS)

FIGURE 5.21

Cube root of glucose uptake (mg ml^{-1}x10) against time for ALF run 12 (○) and ALF run 13 (●).

FIGURE 5.22

Glucose uptake (mg ml^{-1}x10) against time for STR run 22 (⊙).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0
1
2
3
4
5
0
1
2
3
4
5
6
7
TIME (DAYS)

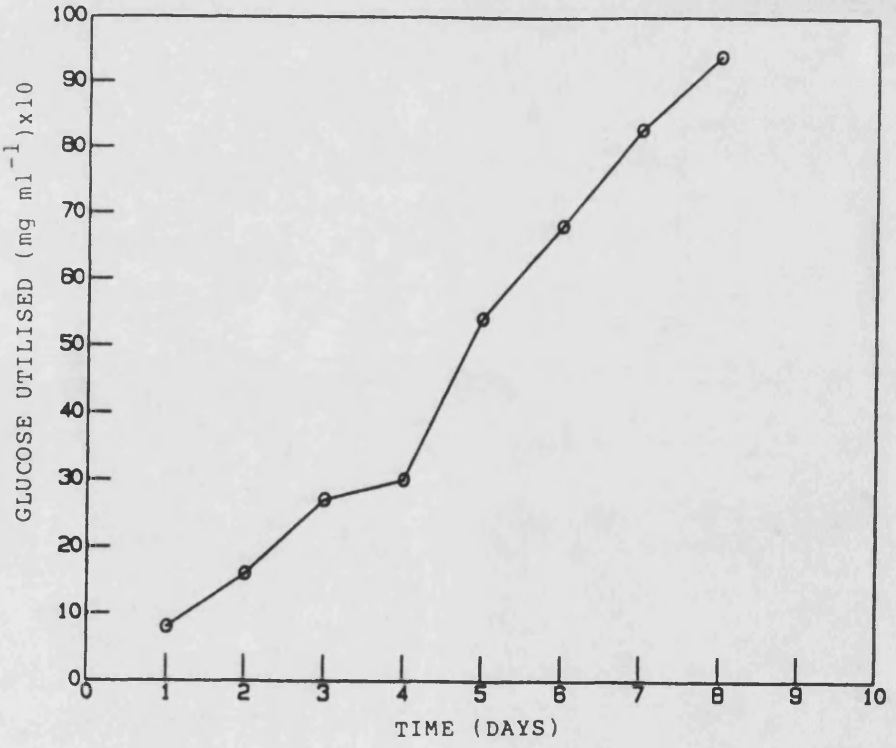

GLUCOSE UTILISED (mg ml^{-1})x10
0
10
20
30
40
50
60
70
80
90
100
0
1
2
3
4
5
6
7
8
9
10
TIME (DAYS)

FIGURE 5.25

The STR (run 10) showing attached growth.

A = Attached growth above the level of the medium.

FIGURE 5.26

The STR (run 10) showing attached growth.

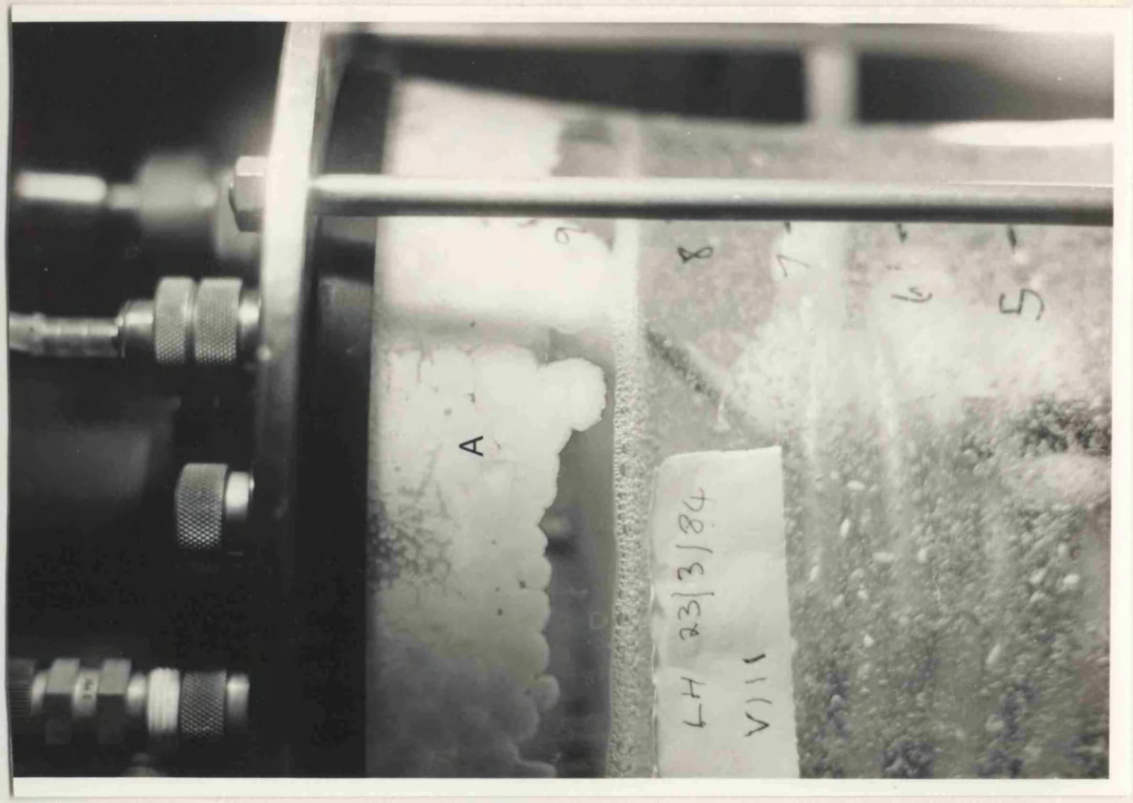
A = Attached growth above the impeller.

B = The impeller.

C = Attached growth between the cooling coil, heater and sparge tube.

D = Heater.

E = Sparge tube.


A
LH 23/3/84
VIII

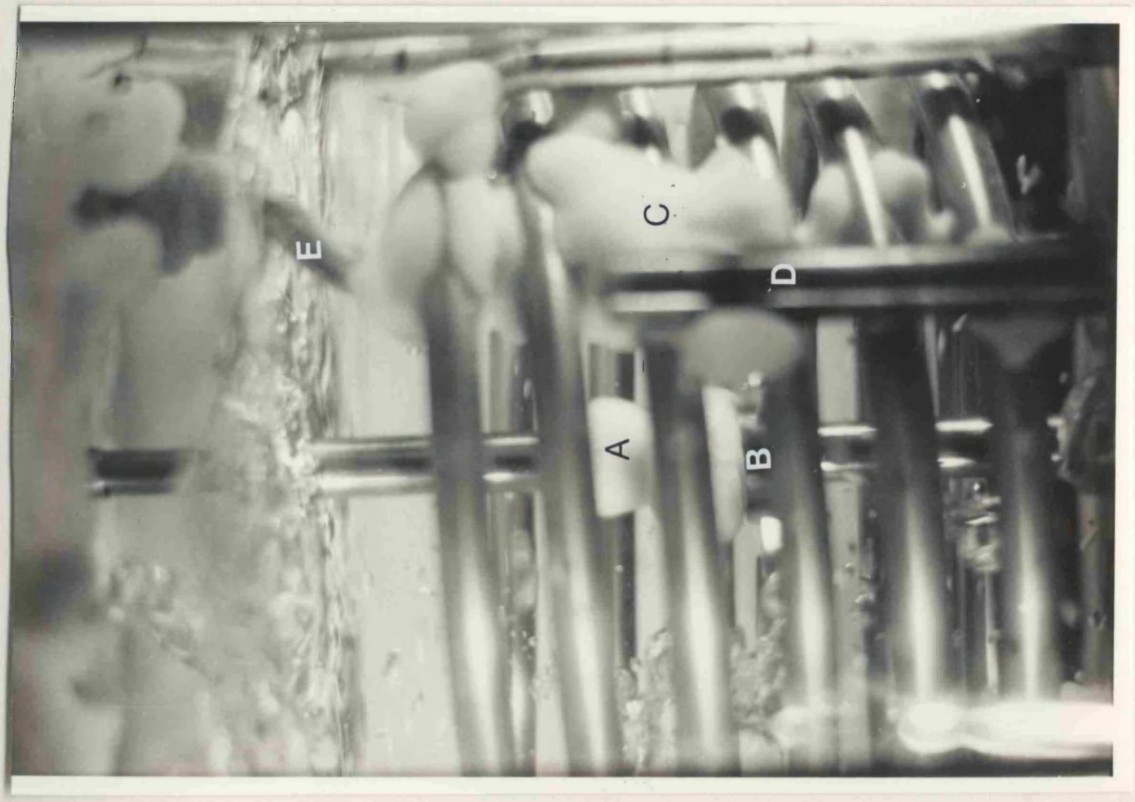

E
C
D
A
B

FIGURE 5.27

The STR (run 10) showing attached growth.

A = Attached growth on the temperature probe.

FIGURE 5.28

The STR (run 10) showing attached growth.

A = Attached growth on the pH probe.

B = The pH probe.

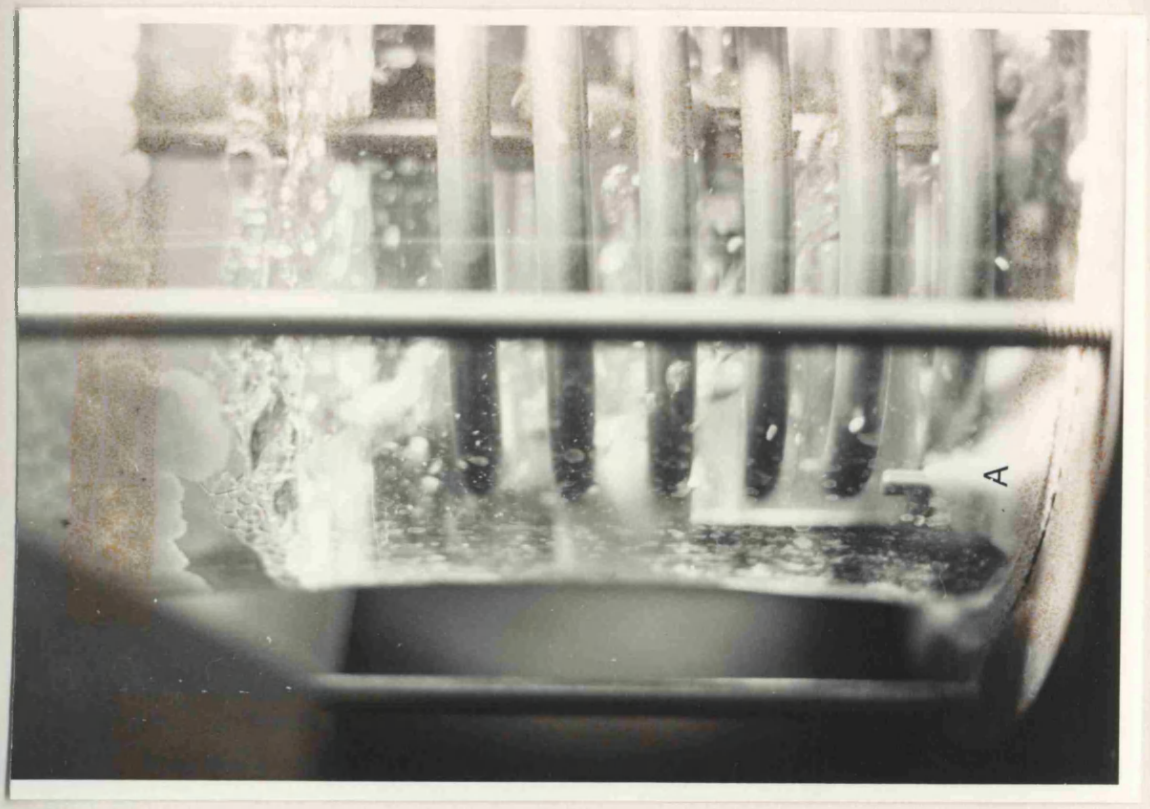
C = Attached growth between the cooling coil, heater and sparge tube.

D = Heater.

E = Sparge tube.

F = Growth between the oxygen probe and the vessel wall.

G = Oxygen probe.


A

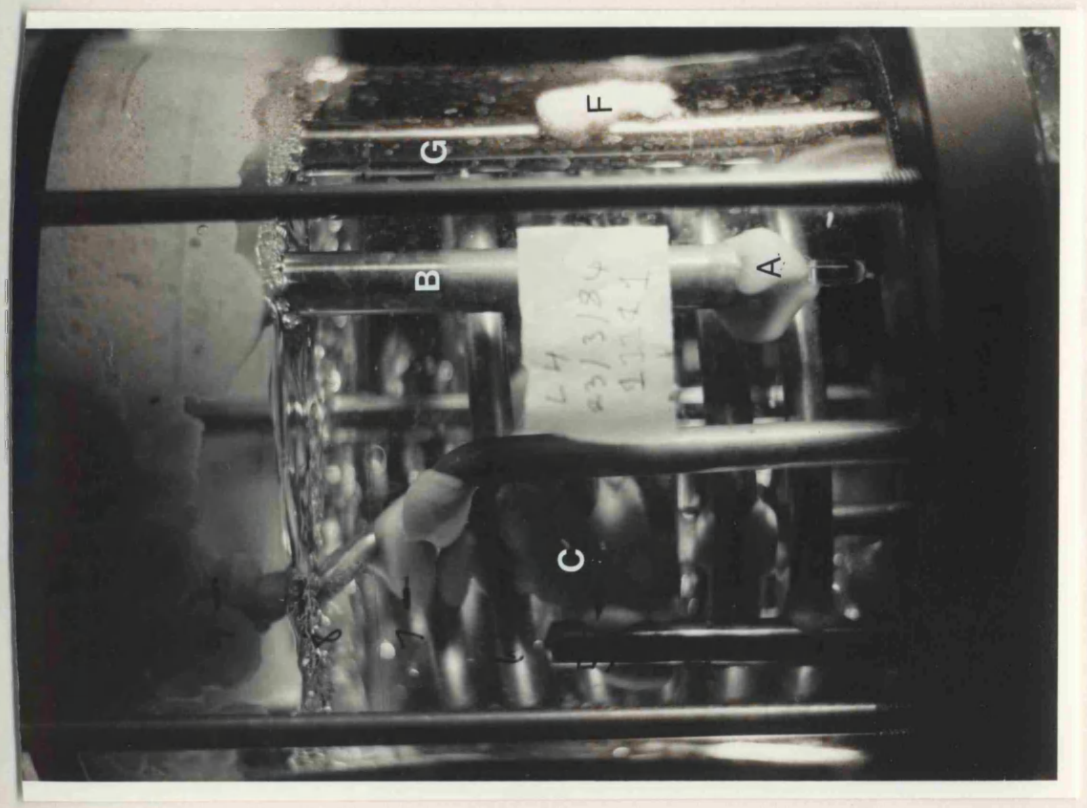

F
G
B
A
C

FIGURE 5.29

The STR (run 11) showing suspended growth, the small white dots in the photograph are pellets.

FIGURE 5.30

The STR (run 11) showing attached growth at the bottom of the fermenter. The small white dots are pellets.

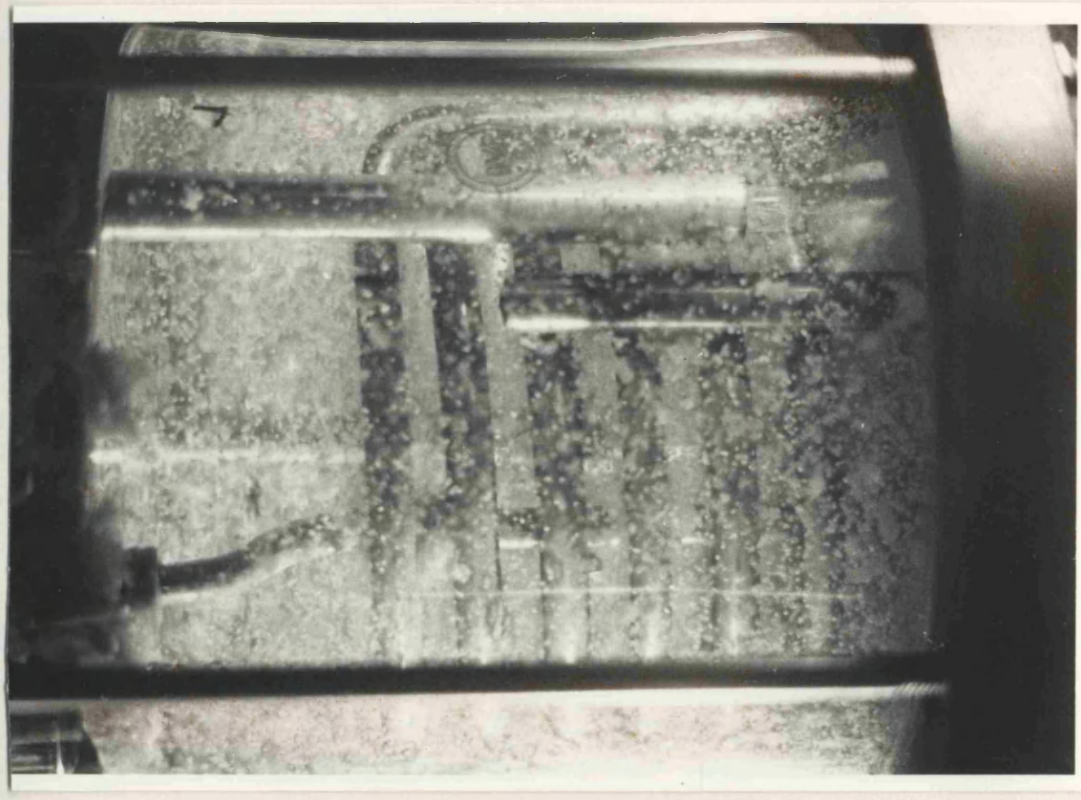
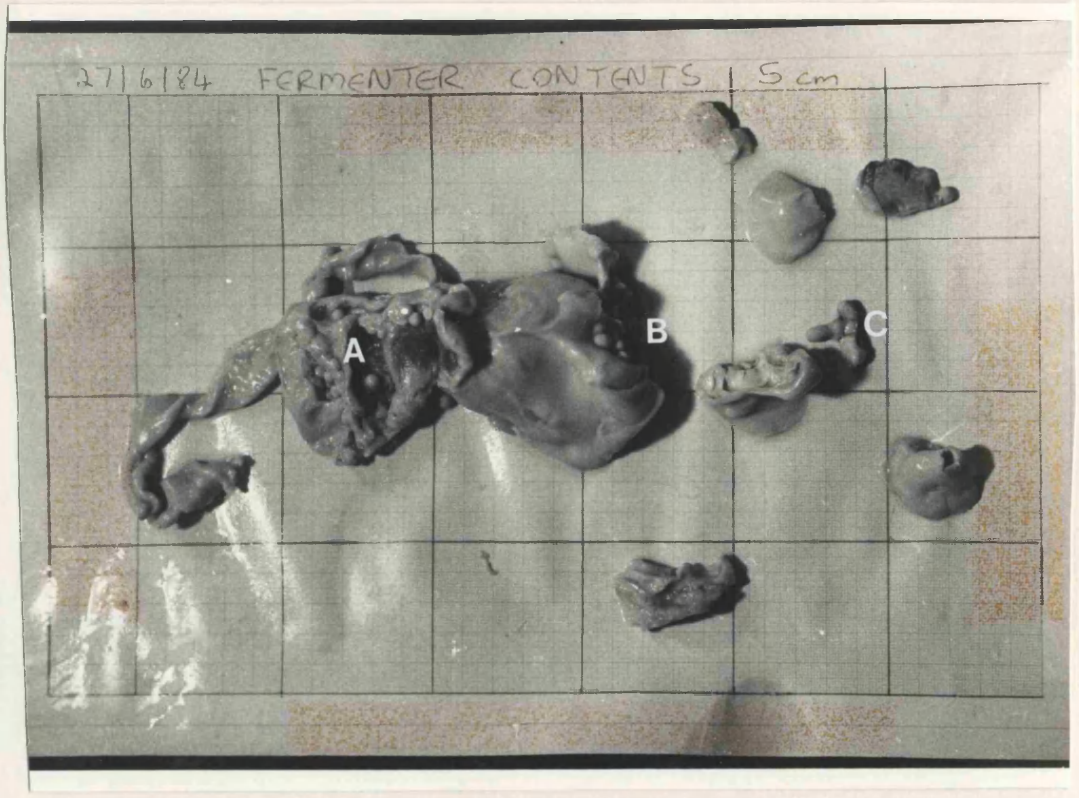


FIGURE 5.31

The STR (run 11) showing the total attached growth. A,B and C are all areas which appear to contain accumulations of pellets.

FIGURE 5.32

The STR (run 11) showing the total attached growth.
A = Hollow area within the fungal mass.


27/6/84 FERMENTER CONTENTS 5 cm
A
B
C

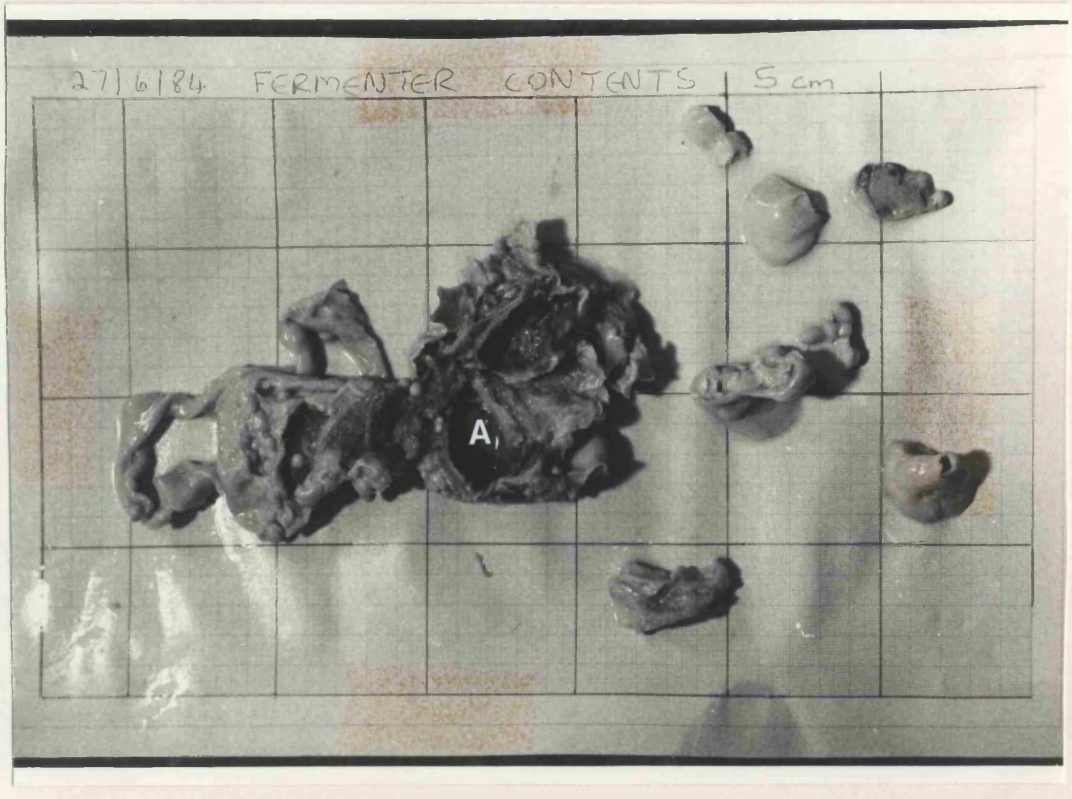

27/6/84 FERMENTER CONTENTS 5 cm
A1

FIGURE 5.33

The ALF (run 5) showing attached growth.

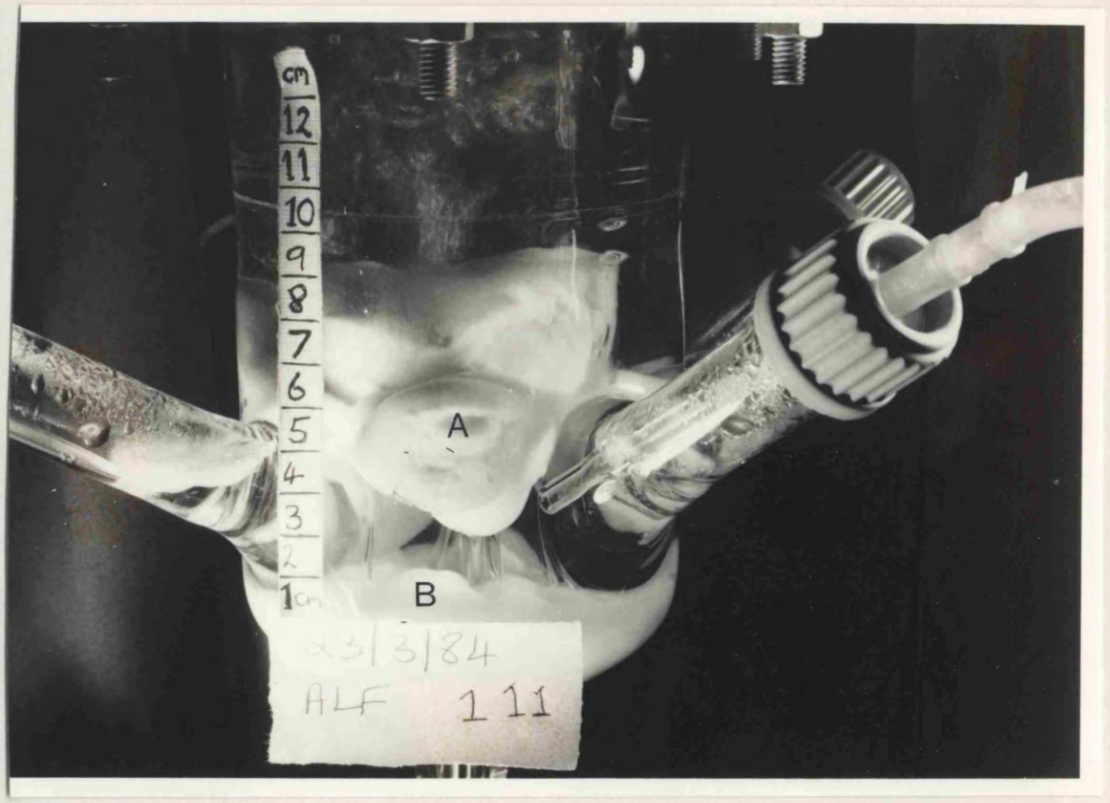
A = Attached growth at the bottom of the draught tube.

B = Attached growth at the bottom of the fermenter.

FIGURE 5.34

The ALF (run 5) showing attached growth.

A = Attached growth on the draught tube top support.


cm
12
11
10
9
8
7
6
5
4
3
A
B
23/3/84
ALF 111


ALF
V
A
cm
10
9
8
7
6

FIGURE 5.35

The ALF (run 6) showing suspended growth. The white particals in the medium are pellets.

FIGURE 5.36

The ALF (run 6) showing pelleted growth.

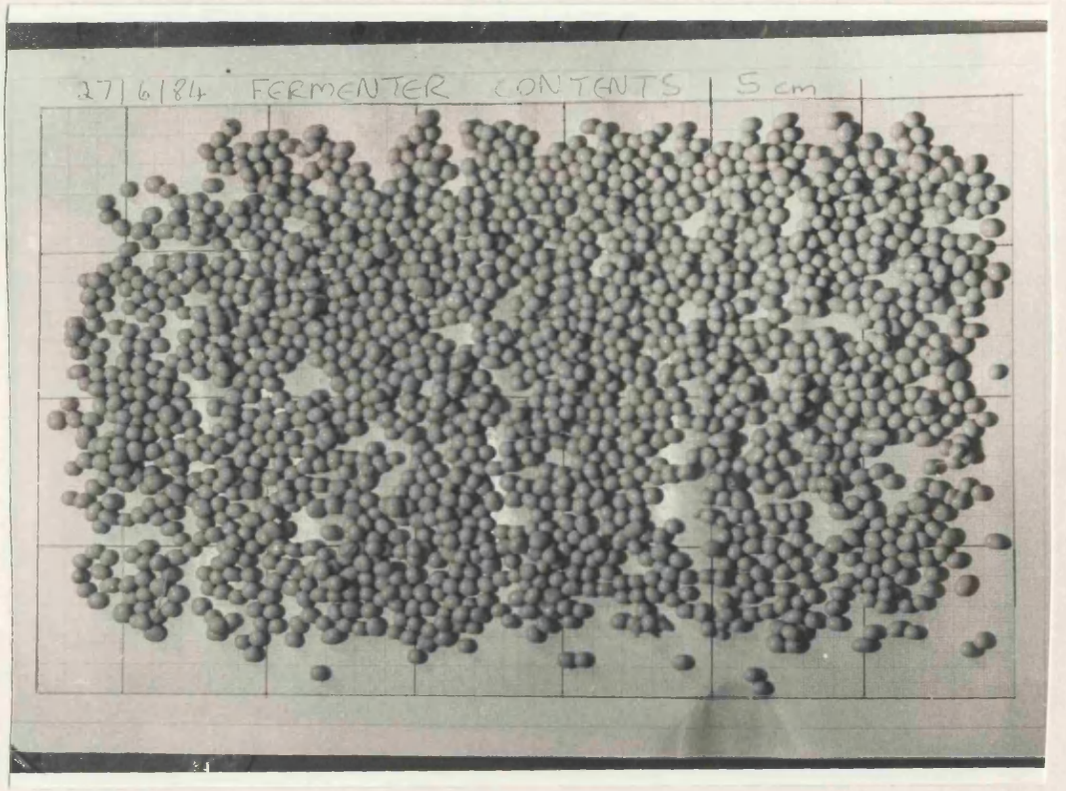
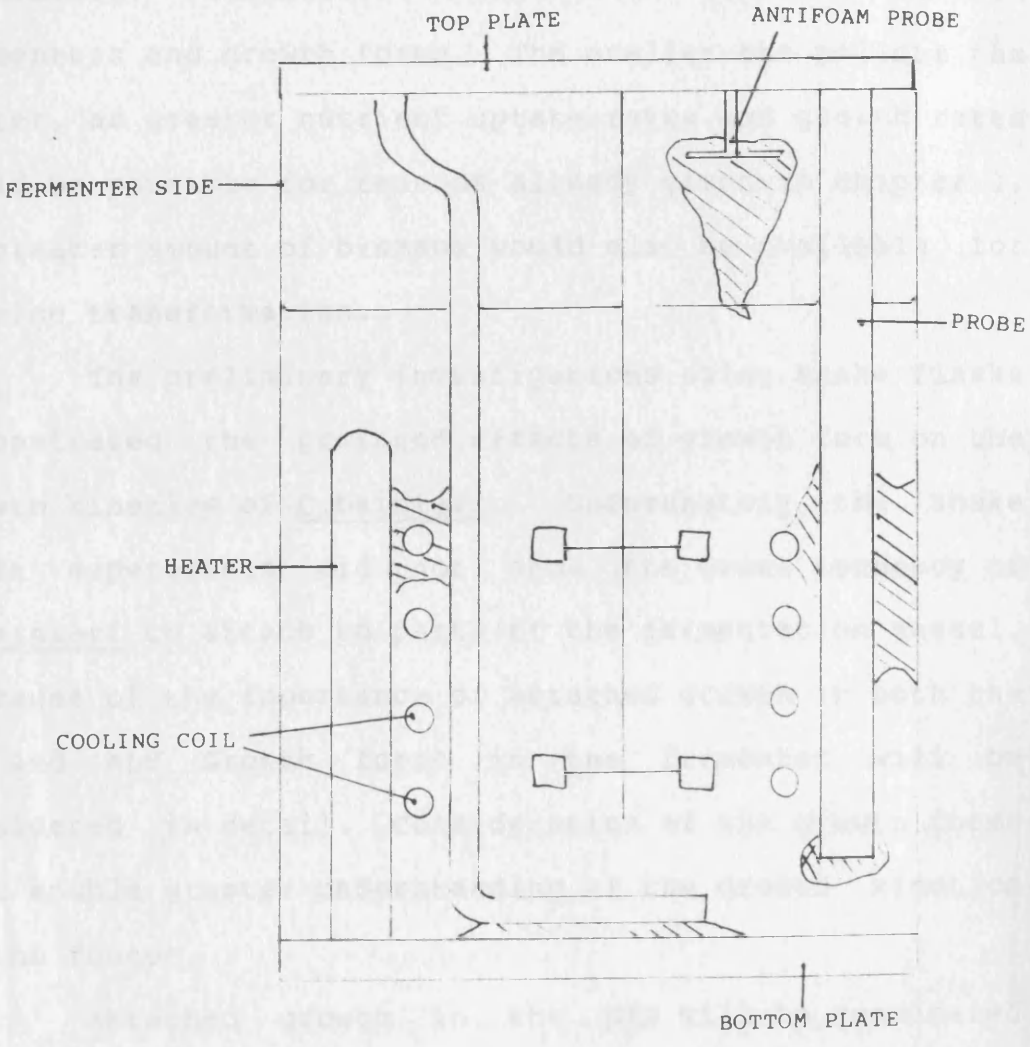

27|6|84 FERMENTER CONTENTS 5 cm

Figure 5.37

A Schematic Diagram of the STR Showing the Main Points of Fungal Attachment. Hatched Areas Represent Fungus



5.4 The Effects of Fungal Growth on the Fermenters

5.4.1 Introduction

The aim of the fermenter experiments was to produce regular pelleted and/or mycelial growth in both fermenters. Comparisons could then be made between fermenters and growth forms. The smaller the pellets the better, as greater nutrient uptake rates and growth rates would be possible for reasons already given in chapter 1. A greater amount of biomass would also be available for codeine transformation.

The preliminary investigations using shake flasks demonstrated the profound effects of growth form on the growth kinetics of C.bainieri. Unforunately the shake flask experiments did not show the great tendency of C.bainieri to attach to parts of the fermentation vessel. Because of the importance of attached growth in both the STR and ALF Growth forms in the fermenter will be considered in detail. Consideration of the growth forms will enable greater understanding of the growth kinetics of the fungus.

Attached growth in the STR will be considered first because the design of the ALF was affected by early STR experiments. The forms of growth observed in the fermenters are described.

5.4.2 The STR

Attached growth was the norm in the STR and pelleted growth was normally of little significance. No filamentous growth was observed. Diagrams and photographs illustrate the extent of attached growth (figs 5.25-5.32, 5.37). It is necessary to complement the above illustrations with written descriptions of the effects of attached growth on the different parts of the fermenter. Some of the points of attachment could be removed, or altered, to reduce the incidence of attached growth. As will be seen this merely shifted attachment to other sites.

5.4.2.1 Air Sparger

If the air sparger was used on intermittent feed it became blocked with fungus. The end of the sparger was closed with a screw and this point invariably acted as a focus for attached growth. The gap between the fermenter base plate and the sparger bottom was often blocked with attached growth (fig 5.37). Intermittent sparging was stopped and the blockage of the sparger tube was eliminated. However, attachment at the other two sites could not be prevented.

A similar blockage of tubing occurred in the sample tubes which were subsequently removed.

5.4.2.2 Inserts Which Are Near the Cooling Coil

Many of the inserts used in the fermenter were

close to the cooling coil. Pellets of fungus were trapped at the points where the coil and intrusions were closest. Once immobilised the pellets continued growing and may have acted as collecting points for even more pellets. The inserts which were observed trapping pellets were the pH probe, oxygen probe, sample tubes, heaters and sparger.

5.4.2.3 Baffles

Baffles were only used in two fermentations because they collected very large proportions of biomass from the medium. Very small particals accumulated in the joints between the spot welds on the baffles. larger particals jammed between the wall of the vessel and the baffles. In the second fermentation no free floating material remained after 2 days. Removal of the baffles led to the displacement of the attached biomass to other anchor points.

5.4.2.4 Foam Detector

The foam detector was rendered useless by attached fungal growth. When placed close to the surface of the medium the detector developed a thick coating of fungus. The fungus trailed into the medium and continually triggered the antifoam pump.

5.4.2.5 Electrode Tips

Both the pH electrode and the oxygen electrode

suffered from attached growth which anchored at gaps between the electrodes and the tips of the probes.

5.4.2.6 Gaskets

The bottom gasket acted as an anchor point for fungal spores. Substantial proportions of the fungal biomass in the fermenter were found as a ring around the bottom of the fermenter.

5.4.2.7 Other Parts Of the Fermenter

The portions of the glass fermenter body continually rinsed by medium were clear of attached growth during the fermentation. Everything above this level was covered in a fine layer of mycelia. This layer was very difficult to dislodge by a transient increase in stirrer speed. PTFE spray helped the removal of the fungus.

Parts of the top plate which came into contact with the medium became very heavily overgrown.

5.4.3

Airlift Fermenter

Both attached and pelleted growth were observed to occur in the airlift. No mycelial growth could be initiated. The amount and siting of the attached growth was related to the internal organisation of the fermenter components. Initial runs were mostly dominated by

Figure 5.38

Fungal Growth on the Draught Tube Top Support

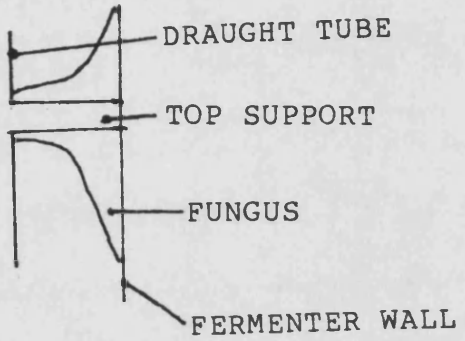
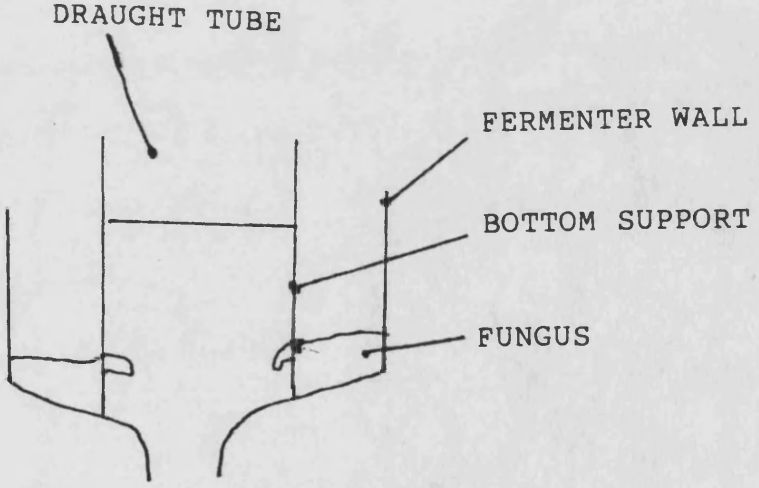


Figure 5.39

Fungal Growth Around the Draught Tube Bottom Support



attached growth (figs 5.33,5.34) but later runs were either mixed or dominated by pelleted growth (figs 5.35,5.36). Attached growth will be discussed first.

5.4.3.1 Draught Tube Bottom Support

The first draught tube design had a 1 cm high support ring to make the glass legs more rigid. Fungus rapidly accumulated between the fermenter wall and the ring (fig 5.38). This accumulation could not be prevented by increasing the air flow rate from 5 l min^{-1} to 10 l min^{-1}. Halving the height of the gap between the draught tube and the ring from 10 cm to 5 cm was also inneffective. It was hoped that these measures would increase the turbulence at the bottom of the fermenter and prevent settling of the fungus. Once the fungus grew level with the top of the ring it grew inwards towards the sparger. The removal of the ring resulted in the loss of a major attachment site for the fungus.

5.4.3.2 Tripod Legs

Attachment was limited to the points at which the legs were buffered from the fermenter wall by a thin layer of silicon rubber compound. The new design also enabled the resuspension of settled any material, using a 1 minute increase in the gas flow rate.

5.4.3.3 Draught Tube Bottom

During some fermentations growth began at the

junction of the draught tube bottom and its supporting legs. This growth could not be dislodged during the fermentations and could not be prevented by spraying with PTFE. Close observation showed that this attachment was caused because long thin filaments wrapped around the draught tube supports. In four cases this growth resulted in the complete blockage of the draught tube (fig 5.33). Once started the growth was rapid and supplemented by the accumulation of pellets from the medium.

5.4.3.4 Draught Tube Top Supports

Small blobs of silicone rubber compound were used to protect the fermenter wall from scratching by the supports. Attached growth began at the junction between this compound and the fermenter wall. All fermentations exhibited attached growth at these sites but the extent of growth varied. In cases of substantial growth pellets accumulated behind the supports. This was presumably caused by the formation of a quiescent zone with very low shear directly behind the support. Air bubbles were retained for long periods in the volume just behind the overgrown supports. Accumulation of pellets on the supports caused a rapid increase in size of the obstruction at the top of the fermenter.

As a result of the obstruction the liquid velocity in the downcomer dropped. This led in turn to a build up of pellets at the bottom of the fermenter and

the effective end of the fermentation. The shape of the growth on the top supports was tapered, the widest part being at the junction with the fermenter wall (figs 5.34,5.39).

Occasionally the normal growth on the supports was supplemented by strands of fungus.

Using a sheath of silicon rubber for the top supports did not change the growth pattern already described.

5.4.3.5 Downflow Section

Pellets of 2 cm diameter became stuck between the draught tube and the fermenter wall. As with the draught tube supports the jammed pellet could act as a focus for pellet accumulation. Any accumulation of pellets in this area was detrimental to the functioning of the fermenter for the same reasons as the growth on the draught tube supports.

5.4.3.6 Spargers

The single orifice sparger was the only sparger not to gather attached growth.

A small amount of growth normally appeared around the base of the four orifice spider. Some of this could be resuspended by twirling the sparger.

The sintered sparger invariably became the site for the growth of a thick layer of fungus. After two or three days the sparger became overgrown and after another

two days some of the air flow from the sparger was redirected up the outside of the draught tube. When used in conjunction with draught tubes with bottom rings the whole bottom of the fermenter was covered with a layer of fungus much more than 1 cm thick. Uneven flow of air through the sinter was a major factor in this process. A maximum of six to ten air release points were counted on the sparger. Once small particles became lodged in the sinter it was impossible to move them.

5.4.3.7 Side Arms

Rings of fungus grew on the surface of the stagnant liquid in the side arms. These represented a very small percentage of the biomass in the fermenter.

5.4.3.8 Probes and Sampler

The probes suffered in a similar manner to those in the STF. The sampling procedure used prevented the accumulation of fungus within the sampling tube.

5.4.3.9 Joints Between Glass Sections

A small ring of fungus always grew at the joint between the two glass fermenter sections. This did not appear to have any significant effect on the fermenter performance.

5.4.3.10 Air/Medium/Glass Interface

A ring of fungus similar to the ring in shake

flasks sometimes grew. In one case when the liquid level in the fermenter dropped quickly an almost solid crust of accumulated pellets was formed. Spores and pellets were often stranded above the level of the ring.

5.4.3.11 Pelleted Growth

Alterations which reduced sites for attachment of fungus tended to increase the numbers of pellets grown in the ALF. The most important changes were the removal of the draught tube support ring, the reduction of the gap between the draught tube bottom and the fermenter bottom from 10cm to 5cm and the use of a non sintered sparger. The use of a dense inoculum derived from mycelial mats also encouraged pelleted growth.

5.4.3.12 A Comparison of growth in the ALF and STF

Figures 5.1 to 5.24 show that in general the glucose uptake in the ALF was more rapid than in the STF. The shape of the glucose uptake curves were normally similar for the ALF and STF. Pelleted growth occured to some extent in most of the ALF fermentations. The large surface area of these pellets explains the more rapid glucose uptake than in the STF. However, the attached growth in the STF presented a potentially greater surface area per unit volume than attached growth in the ALF (figs 5.25-5.34). Attached growth also grew as a section of a sphere. These factors explain why the difference between the fermenters was not great and why a cube root

relationship between glucose uptake and time could be detected for both fermenters (eg figs 5.10-5.12).

An inoculum of spores, mycelia and agar preincubated for at least 4 hours after which the liquid and suspended matter (but not the mycelial mat) were added to the fermenters provided the most rapid growth.

Total glucose utilisation was rarely achieved during the course of the fermentation. Compared to the shake flask fermentations cultures in the fermenters were slow growing, but not as slow growing as the occurrence of attached growth suggested. The size of pellets and attached lumps of fungus made the reason for this slow growth obvious, a small surface area of fungus was available for growth and nutrient uptake.

Antifoam did not affect the occurrence of attached growth.

5.4.3.13 Codeine Conversion

The occurrence of codeine conversion was rare as can be seen from the results in figure.

5.5 Conclusions

5.5.1 General

The ALF was shown to be capable of being used as a culture vessel for C.bainieri. It suffered from many of the same problems as the STF but was much more responsive to alterations than the STF. By altering the

physical aspects of the fermenter, such as the size of the gap at the bottom of the draught tube, a considerable decrease in the incidence and size of attached growth occured. It was seldom possible to maintain any quantity of the fungal biomass in the STF as suspended pellets, in the ALF on the other hand a substantial proportion of the fungal biomass could be present as pellets. This is reflected in the consistently more rapid glucose uptake rates for the ALF compared with the STF even when other factors were altered.

Total glucose depletion could be achieved after 5 to 6 days of fermentation. This compared favourably with glucose uptake by shake flask cultures which could deplete glucose after 4 days when inoculated with spores.

5.5.2 Growth Kinetics

A few generalisations can be drawn from the growth kinetics exhibited by the fermenters in paired experiments.

1) The type of inoculum was obviously important. The fermenters run in pairs usually showed similar growth rates.

2) Growth in the airlift was normally more rapid than in the STF.

3) Pelleted growth was rare in the STF but this did not hinder nutrient uptake as much as may be expected.

4) Small pellets led to a more rapid uptake of glucose

5) Pregerminated spores produced a more rapid increase in glucose uptake than the spores added directly to the fermenters.

6) The low level of codeine conversion detected was probably due to the slow depletion of glucose and the relatively small surface area of the fungus in the fermenters.

5.5.3 Airlift Fermenter Experiments

The airlift fermenter was used to assess the effects of different conditions on the growth of C.bainieri and vice versa. Liquid loss and physical blockage of the fermenter were the main problems associated with the airlift. At the air flow rates used, antifoam was not necessary to control foaming.

One of the problems with fermenter design is that the results of shake flask experiments may not be directly transferrable to larger fermenters. This problem was very well illustrated during the course of this project. Shake flask experiments provided a lot of useful information about the growth of the fungus and the best inoculum procedures.

Growth in shake flasks was primarily suspended with attached growth contributing relatively little to the overall growth in the flask. Attached growth could be resuspended on a regular basis. Although this

probably had an effect by increasing pellet size and decreasing pellet number the glucose in the medium was usually depleted rapidly. The only clue that the fungus might cause problems was that the pellets did not fragment after formation, even several weeks after total glucose depletion.

In the more complex vessels the attached growth was difficult to remove and therefore of much greater importance. This was for three reasons.

Firstly the biomass growing on pH and oxygen probes rendered them useless. By virtue of their design, especially when contained in the pressure housings used on the STF the probes proved to be excellent sites for attachment. This had serious implications for the control of the fermenters.

Secondly the quantity of attached growth seriously reduced the glucose uptake rate.

Thirdly the presence of attached growth reduced the number of suspended pellets. A reduction in medium circulation rate, disruption of flow patterns or mechanical capture caused by the narrowing of the gaps between parts of the fermenter all caused a decrease of suspended material. Attached growth reduced the number of suspended pellets.

5.5.3.1 ALF Growth forms

a) Pelleted Growth

Pelleted growth in ALF's is encouraged by

removing dead spaces, ensuring adequate medium circulation, avoiding the use of non germinated spore inoculum and using non sintered spargers. As the section on shake flask experiments shows germinated C.bainieri spores form small pellets soon after germination. By using pre germinated spores it was hoped to avoid attachment of single spores to surfaces by using pores which were already agglomerated. This tactic met with some success but could not prevent attached growth from causing the removal of most pellets after 4 to 8 days.

When unhindered pelleted growth did occur the growth curves for the fermenter were much more rapid than when attached growth was dominant (figs 5.19-5.21).

It seems likely that in a larger fermenter the importance of attached growth would be much reduced.

b) Attached Growth

Attached growth can occur at many points in the ALF. Some of this growth is quantitatively insignificant, but in the case of electrodes qualitatively important. Control of fermentations which give varying results not related to culture conditions was very difficult.

Large accumulations of C.bainieri were normally found around the draught tube supports and at the bottom of the fermenter. Removal of the draught tube support ring prevented the accumulation of large amounts of biomass around the sparger. However, the site of

attachment tended to shift to the area around the draught tube supports. Often the size of the attached growth was augmented by the removal of fungal pellets from the medium. This accumulation led to a rapid decline in medium circulation rate and settling of pellets to the bottom of the fermenter. The loss of more suspended material led to greater inhibition of medium circulation. An increase in foaming occured and the run had to be terminated. Examination of the fungal masses at the end of the run confirmed that pellets agglomerated to form attached growth.

Growth on the sintered sparger had a markedly deleterious effect on liquid circulation after about 5 days when diversion of air up the outside of the draught tube became noticeable.

Attached growth at the bottom of the fermenter could be largely prevented. The lowering of the gap between the draught tube from 10cm to 5cm, removing the glass ring from the bottom of the draught tube and the use of a non sintered sparger achieved elimination of dead spaces. As a result attached growth was prevented. However, no sure method of preventing attached growth was found.

c) General Comments on the Airlift Fermenter Design

The construction of most of the fermenter from glass produced some of the problems involved with the fermenter. Top and bottom supports for the draught tube

were prone to breakage, as were the fermenter side arms.

No breakage of draught tube supports occurred until the ring at the bottom of the supports was removed. Once the ring was removed the legs became prone to breakage during the journey to or from the steam line. Spare sections were made for the ALF but time loss due to breakage after sterilisation could be several days. This was because of problems in ensuring a reliable steam supply.

Care had to be taken when fastening the sections of the fermenter together to minimise the possibility of damage to the side arms. Repeated heating and cooling caused the screw tops on the side arms to become stuck and attempts to unscrew them could lead to breakage.

The gap between the bottom of the draught tube and the fermenter bottom could not be closed enough to prevent small dead spaces forming. These acted as a trap for pellets and spores. Redesigning the bottom section so that it sloped upwards towards the sparger would prevent this dead space.

Several ALF runs had to be stopped because of liquid loss. It would be advisable to use a non wettable filter for air inlet and outlet filtration. A saturated air supply at 27 ^{o}C could then be used for aeration.

The growth of fungus around the top supports of the draught tube could cause considerable restriction of flow. This was partially overcome by raising the supports so that they rested in the disengagement area.

5.5.3.2 STF

Control and monitoring of the STF was rendered almost useless by fungal overgrowth on the probes. No method of preventing growth on the probes was discovered, although there are techniques to deal with the overgrowth of probes such as sterile replacement of probes during the fermentation it was not possible to use them. Most of the fermentations resulted in attached growth with very little pelleted growth. A coating of PTFE helped prevent attachment on the fermenter walls but had no great effect on the total amount of attached growth. Fermentations with large quantities of attached growth resulted in slow glucose uptake.

Attached growth could be reduced by removing obstructions. Some of the other recommended methods such as increasing the medium volume did not work. Increased volume led to the attachment and growth of fungus on three inserts protruding from the top plate.

Very little pelleted growth occured in the STF. The pellets were consistently smaller in diameter than in the ALF but pelleted growth gave a very similar glucose uptake to ALF pelleted cultures.

5.5.4 Summary

Shake flask, ALF and STF experiments suggest that:-

i. Attached growth was not due to any ability of the grown fungal pellets to stick to surfaces other

than C.bainieri. Most of the attachment appeared to be a result of the mechanical accumulation of biomass. This was originally as filaments, spores or pellets of a few spores. As can be seen from the photographs in the results section the attachment of fungus only occurs in dead spaces or points at which the filaments can wrap around a support. Once growth was initiated and the fungus had encircled its attachment point detachment was impossible. Pellets were removed from the culture medium by the initial attached growth. The removal of suspended growth was caused by the creation of dead spaces by the closing of gaps between fermenter components or by entrappment of pellets in the narrowed gaps. The pellets then became incooperated with the attached growth (figs 5.31,5.32).

ii. Medium components probably had an effect on the likelyhood of pellet formation. Lumps of used malt extract agar (chapter 2) or undefined medium used by Sewell (97) caused filamentous growth of C.bainieri.

iii. Pellets and attached growth were never seen to disintegrate as a result of senescence.

iv. Changing the agitation rate in the STF did not increase or decrease the amount of attached growth observed

v. The STF possessed many sites for the attachment of

v. The STF possessed many sites for the attachment of pellets.

vi. No inoculum technique would reliably produce pelleted or filamentous growth in fermenters.

CHAPTER 6

6.1 CONCLUSIONS

6.1.1 Main conclusions

6.1.1.1 Fermenter type

The ALF was the better bioreactor for the growth of *C. bainieri* than the STR for the following reasons:

a) Large numbers of pellets could be produced in the ALF using spore inoculum. This was not the case in the STR, the removal of one attachment site resulted in attachment elsewhere (Sections 5.5.3, 5.5.4).

b Glucose uptake in the ALF was more rapid than in the STR (eg Figs 5.7, 5.10, 5.13, 5.16).

6.1.1.2 Increase in Fermenter Size

The increase in scale from shake flask to ALF and STF scale resulted in a decrease in norcodeine production and the rate of glucose uptake.

This result was almost certainly due to the reduction in surface area per unit biomass of fungal clumps which occurred in the fermenters.

The fermenters used could be ranked in the following order - shake flask, ALF, STF.

In general large scale culture did not perform as well as, or in, the manner desired (see Chapter 1). This however, was an attempt to solve the problem of culture of *C. bainieri* in

suspension. It is very likely that an immobilised system would produce better glucose uptake and codeine conversion rates. This is because particle size can be controlled and attached growth reduced considerably. Hence better environmental control can be linked with smaller particles.

6.1.1.4 Fungal Strain

C. bainieri was suitable for suspended growth in shake flasks but less suited for growth in larger vessels. A programme of mutations and selection would be of great benefit for the following reasons:

a) To increase yield of norcodeine

b) It is possible to produce strains of fungi which exhibit different morphologies in submerged culture. Selection of a suitable strain should prevent massive attachment of fungus to surfaces within the fermenters.

6.1.2 Conclusions - scale up

Attached growth was the predominant growth form in both the ALF and STF independent of the type of inoculum used and agitation rate (Section 5.4). The removal of attachment points for fungus the STF resulted in the attachment of C. bainieri to the remaining attachment sites. An increase in pelleted growth was achieved in the ALF and resulted from the lowering of the draught tube and the removal of its support ring (5.5.1 p 233-234). This suggested that pelleted growth should be possible in either vessel if sites of attachment could be eliminated.

Unfortunately, this could not be achieved in the STF.

Consequently, one of the aims of scale up - to produce a culture similar to those in shake flasks with a large number of small pellets was partially successful in the ALF but unsuccessful in the STF.

6.1.2.2 Oxygen Transfer

In both fermenters when the oxygen electrode was not covered by fungus the dissolved oxygen tension in the medium remained constant. Therefore, the outer portions of the fungal masses were not oxygen limited (Section 1.6).

6.1.2.3 Agitation

The use of high stirrer speeds to reduce filamentous growth in the STF only produced uncontrollable foaming. As little suspended growth was detected at stirrer speeds of 1000, 700 and 500 rpm the effect of tip shear rates on attached mycelia was insignificant. The few pellets present were smaller than those in the ALF (Sect 5.5.3.2, p 240). Therefore even at low stirrer speeds in an unbaffled STR stirring affects pellet growth. Hence one of the differences between ALFs and STRs is demonstrated. Any activity such as gassing or stirring which caused splashing of the medium caused the growth of fungus above the level of the medium.

6.1.2.4 The Effect of Attached Growth on the Fermenter

Attached growth in the ALF exhibited slower growth

kinetics than pelleted growth. Liquid circulation in the ALF was seriously affected by attached growth. Unlike the liquid flow in the STF. The most damaging growth was the attached growth at the top of the draught tube (Fig 5.33) and the top supports of the draught tube (Fig 3.34). The effect of growth of attached fungus was like closing a valve. As the circulation rate dropped the pellets could no longer be kept in circulation and they fell to the bottom of the fermenter.

The effect of growth on the draught tube could be reduced by increasing the height and diameter of the fermenter (Section 1.7). As the volume of the reactor would also be much larger this may have the effect of increasing the number of pellets formed by reducing the chance of impact with an obstacle or dead space.

6.1.3 Shake flasks

6.1.3.1 Growth Kinetics

There was no direct relationship between the spore concentration and growth kinetics of C. bainieri. To some extent higher spore concentrations in the medium resulted in greater glucose uptake in the culture as would be expected (more biomass). However, the increase in uptake was difficult to quantify because of the variability between replicates. Other factors such as the presence of surface active agents or spent malt extract agar appeared to have more relevance. This was amply demonstrated in runs IE1 to IE3 (see Chapter 3).

6.1.3.2 The Usefulness of Averaged Data

Variability between the shake flasks such as seen in virtually all the shake flask experiments indicate that individual fermentations must be followed to extract meaningful information about the growth of the fungus under these conditions. Antifoam experiments AF1 and AF2 provided supporting evidence of several types:

i) In AF1 after 7 days of fermentation all the glucose was utilised in all the flasks of the antifoam B run. Of the flasks in the antifoam C run only three showed total glucose utilisation.

ii) The weight of norcodeine produced could not be directly correlated to dry weight after 7 days of fermentation (AF1).

iii) Codeine conversion could still not be correlated to dry weight after 12 days of fermentation (AF2).

All the inocula produced variable growth results. This finding was amply demonstrated in Chapter 3. The variability included:

i) Differences in the ratio of product to dry weight between replicates (AF1 and AF2).

ii) A range of fungal morphology was observed in sets of replicates (eg IE2).

iii) The rate and kinetics of glucose uptake varied considerably within replicate cultures.

6.1.4.3 Spores as Inocula for Shake Flasks

Experiments IE1, IE2 and IE3 demonstrated that the use of

spores to inoculate shake flask cultures was feasible.

6.1.4.4 Cube root Growth Kinetics

Runs IE1 to IE3 showed that C. bainieri pellets showed kinetics of a type predicted by cube root models. In most of the cultures inspected no simple relationship between glucose uptake rate and time was apparent. The absence of mycelial rings and the presence of pellets with a narrow size distribution coincided with the best fits to cube root kinetics.

Tween 80 and antifoam B decreased the variablity of fungal growth between replicate shake flasks in IE2 and IE3.

6.1.4.5 Spore age

Spore age made a difference to the fungal growth rate presumably as a result of differing viability of spores of different ages. Ten day old spores had a good germination rate and were produced in large quantities.

6.1.4.6 The Effect of Antifoam B on Growth and codeine Conversion

The use of 1000 ppm antifoam B did not have any deleterious effects on the growth or glucose uptake of C. bainieri. As experiments AF1 and AF2 show there were no gross effects on codeine conversion either.

CHAPTER 7

FURTHER WORK

7.1 General

Work could usefully be carried out in four areas. These are yield improvement, manipulation of the growth form different, growth techniques and process optimisation.

7.2 Yield Improvement

Improvements of yield and manipulation of the growth form can be achieved by medium design and genetic manipulation.

The work of Sewell (97) and the spore inoculum work (chapt 3) has shown that a less well defined medium than the one used in the fermenters will produce filamentous growth.

Genetic manipulation can take several forms. At the simplest level mutation and selection techniques can have dramatic effects on the productivity of fungi. Antibiotic production by fungi can be increased by up to 50 fold over 10 to 20 generations (2,3). The great advantage of this system is that a detailed understanding of the system which is being improved is not needed. Very large numbers of isolates would have to be screened for increased activity as mutation is relatively non specific (2). However, this type of technique could certainly be profitably applied.

Genetic engineering techniques may be useful for

increasing production of cyt P450's. These techniques would require a much greater understanding of the N-dealkylation system than the previous technique. Several potential vectors for transfer and expression of genes in fungi are known (37,49,50,51,55,81,116,117). In the immediate future genetic engineering seems to be of less use than mutation, selection or cross breeding of strains. In the more distant future genetic engineering may provide information about the control and production of Cyt P450's. It may be possible to produce strains of fungus which produce only one version of the enzyme rather than a range.

7.3 Growth Form

Genetic manipulation and medium design have already been discussed in the previous section. It may also be possible to manipulate growth form if more work is carried out on pellet formation. A second area of investigation is the design of the bioreactors. If small pellets (0.1 to 1mm in diameter) of fungus could be produced it would be worth trying to optimise the system in which these pellets were grown and suspended. The problem with the large pellets so far produced in both shake flasks and the ALF is the small volume to surface area ratio. Low codeine N-dealkylation rates are almost certainly related to this large pellet size.

Because the growth of the fungus as suspended free floating biomass caused such problems immobilised

culture systems for the production and maintainence of biomass should be investigated. These may consist of circulating particles or fungus growing on a fixed matrix. Suitable systems include gel entrapped mycelia (probably grown from entrapped spores), killed fungal pellets, various foam matrices, glass beads and sintered glass.

Immobilisation of the fungus would probably keep the probes clear of fungus and enable better monitoring and control of the fermentation to be carried out. Previous work and literatue sources both suggest that glucose and dissolved oxygen concentration are very important in regulating the induction of cyt P 450's. It would therefore be a great advantage to keep the immobilised fungus dispersed enough to ensure that the mycelia were all experiencing the same culture conditions.

7.4 Optimisation of the Process

The first step in optimising the N-dealkylation process is to produce the fungus in a finely dispered form which can be monitored reliably. At present the N-dealkylation process could be occurring on the surface of the pellets, just below the surface or near the centre where the mycelia are senescent. Under these conditions measurements carried out on the medium mean very little.

Secondly the medium parameters must be optimised with special attention being paid to glucose

concentration, oxygen concentration, carbon dioxide concentration and pH. The importance of the first three parameters has already been discussed. pH is important because it may effect the solubility of the substrate and productof N-dealkylation.

Thirdly the effect of codeine conversion on the fungus should be studied. The efficiency of codeine conversion is very low, approximately 12% when 1mM codeine is used. Increasing the codeine concentration does not significantly increase the total amount of product. Are products of the reaction such as formaldeyde inhibitory, are problems caused by the solubility of codeine and norcodeine in the cell, are codeine and norcodeine able to pass easily between the medium and mycelia? These questions could be answered using radioactively labelled codeine and cell free systems.

Fourthly any new substrate for N-dealkylation must be optimised anew because the specificities of Cyt P-450's for different substrates varies greatly.

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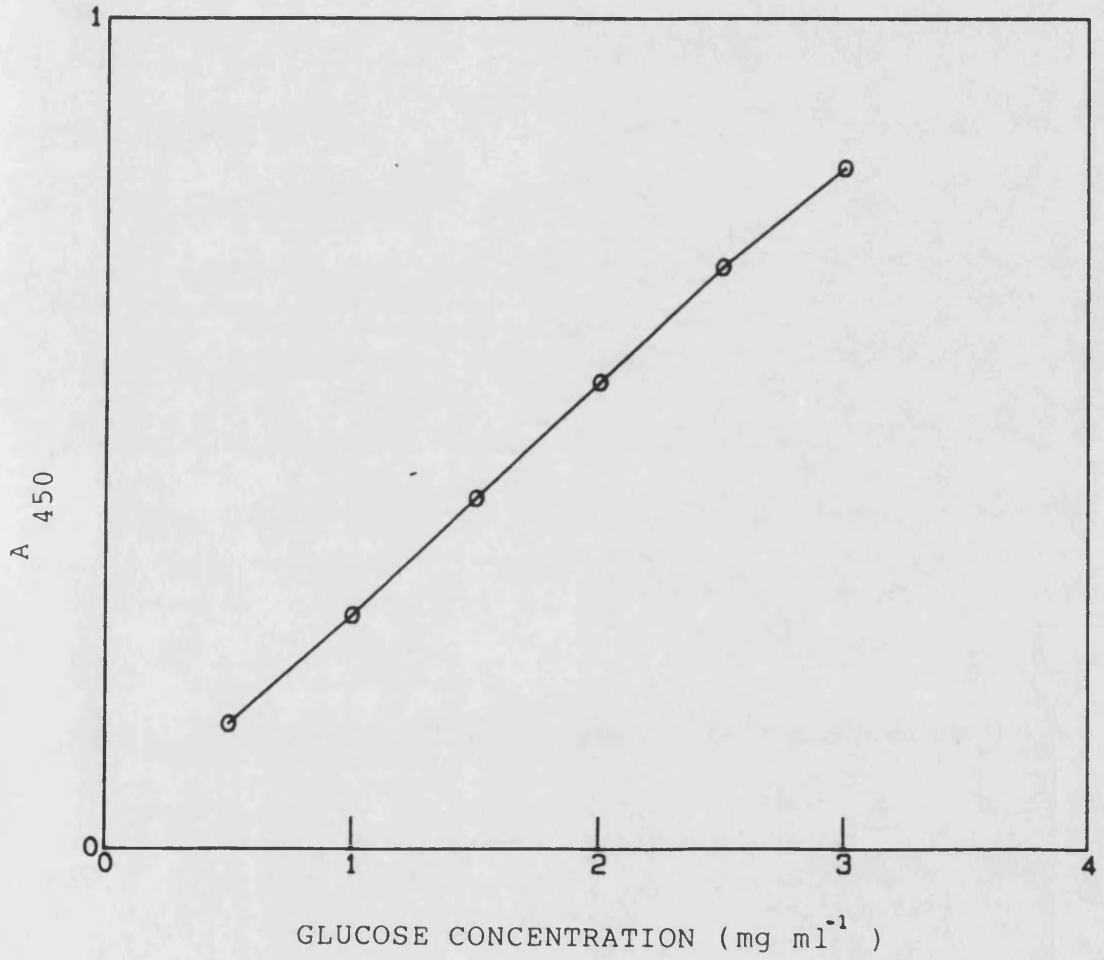
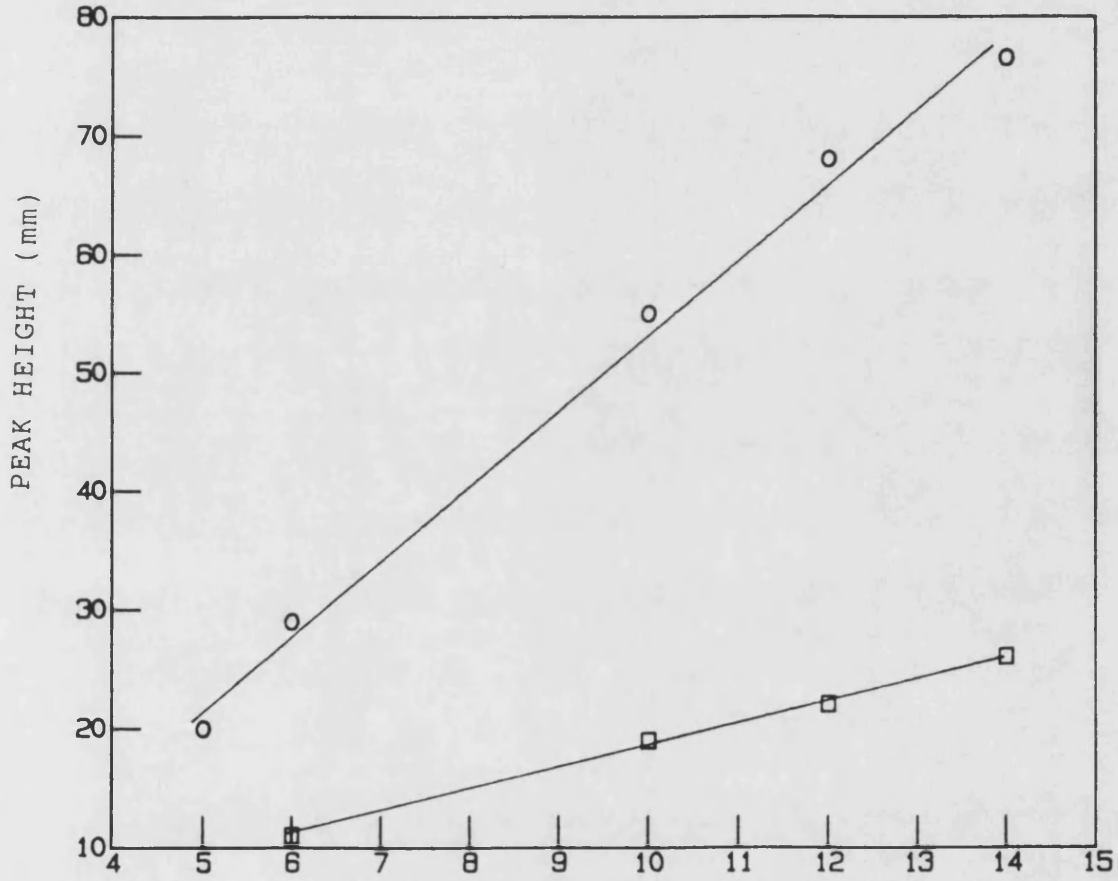
APPENDIX 1

The calibration curve for the glucose assay



APPENDIX 2

The calibration curve for codeine and norcodeine



APPENDIX 3

Sample calculations of the maximum specific growth rate of C.bainieri using shake flask data

Using $X=X_o\ e^{\mu_{max}\ t}$ (96)

Where X= Biomass at time t.

X_o= Biomass at time 0.

μ_{max}= Maximum specific growth rate.

For experiment IE1

Sample 5

Between 6 and 14 hours

X_o= 0.1 mg

X= 26 mg

t= 6-14

$26=0.1e^{8\mu_{max}}$

$260=e^{8\mu_{max}}$

μ_{max}= 0.125 h^{-1}

Sample 6

Between 12 and 16 hours

X_o= 42 mg

X= 125 mg

t= 12-16

$125=42e^{8\mu_{max}}$

$3=e^{8\mu_{max}}$

μ_{max}= 0.35 h^{-1}